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STUDIES ON NEURAMINIDASES

by

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A thesis submitted in fulfilment of the
requirements for the degree of Ph.D. of the
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PREFACE

The work described in this thesis was carried out in the Department of Chemistry and Molecular Sciences, University of Warwick, Coventry, England, during the period between October, 1975 and September, 1978. It is the original work of the author, except where specific acknowledgement is made or implied. This thesis has been submitted at the University of Warwick alone, in fulfilment of the requirements for the degree of Ph.D.

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Finally I am grateful to Jacynth McKeand for her help in typing this thesis.

SUMMARY

In this thesis, the work undertaken in an attempt to gain insight into the catalytic function of neuraminidase is described.

The history, properties and importance of neuraminidases are reviewed in Chapter One. Chapter Two contains an account of the production of neuraminidase from Streptomyces griseus by induction, of its preparation and purification to homogeneity, of its characterisation as a glycoprotein of 32,000 molecular weight and of its structural and catalytic properties. In Chapter Three, chemical modification methods were employed to seek information regarding the nature of the amino acid residues essential in the activity of S. griseus, Cl. perfringens and influenza virus neuraminidases. In all the three enzymes the results obtained suggested that arginine, tryptophan and carboxylic groups were crucial for the enzyme activity. Based on these findings, a mechanism for neuraminidase action was proposed.

The extensively purified neuraminidase from S. griseus was used, in conjunction with an isoelectric focussing technique, to investigate the sialylation differences in human interferons. The results to these experiments, reported in Appendix I, suggest that fibroblast (and not leucocyte or lymphoblastoid) interferon contains neuraminidase-releasable sialic acid residues. The experiments reported in Appendix II, in which rabbits were immunised with colominic acid or fetuin, were conducted to raise antibodies specific to sialic acid in an attempt to design a radioimmunoassay for free sialic acid.

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1. GENERAL INTRODUCTION

1.1 Discovery of Neuraminidase

As early as 1902 Kraus and Ludvig [1] described bacterial haemagglutinins which were destroyed by heating at 58°C ; the ability to haemagglutinate red blood cells was later observed as a property of many protozoa and bacteria. Thomsen [2] and Friedenrich [3] found that, contrary to the high specificity of native human erythrocytes in their reaction with iso-antibodies (isoagglutination), the red blood cells exposed to different bacteria or to their culture fluids are agglutinated by all human sera (panagglutination). They wondered whether this phenomenon derived in a bacterially-caused alteration of the red cell membranes.

About ten years later, Hirst [4] and McClelland and Hare [5] reported that chicken erythrocytes were haemagglutinated by influenza virus. Hirst [6, 7] later showed that, when influenza viruses are allowed to interact with red blood cells at temperatures near 0°C , the viruses remain attached to the cells but are spontaneously eluted from the cells at 37°C . Hirst observed that the red cells from which the virus had spontaneously eluted would neither adsorb, nor become reagglutinated by, freshly added virus. The eluted virus, however, would still reagglutinate fresh cells. Something had changed the surface of the red cells, and Hirst proposed that an enzyme present on the virus particle must have been functional in destroying certain agglutinating receptors on the cells.

Hirst [7] suggested the analogy of the agglutination reaction with the interaction of an enzyme E (on the virus particle) and its substrate S (receptors present at the red cell surface), resulting in the complex ES equivalent to haemagglutination, viz.

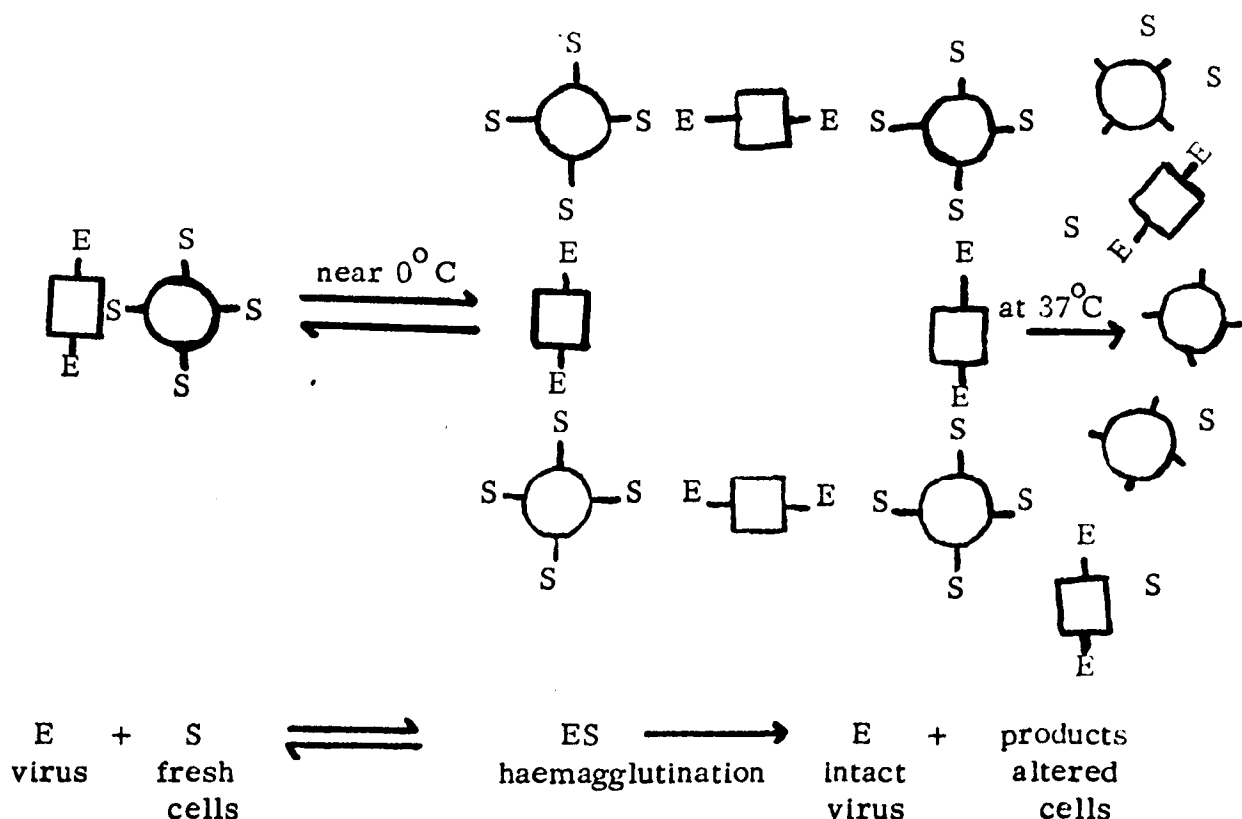


Fig.1.1 Hirst's interpretation of the erythrocyte haemagglutination phenomena

Hirst's postulate contradicted a view previously held that viruses, unlike bacteria or higher organisms, were devoid of enzymes. However, following the discovery that other viruses, e.g. mumps, Newcastle disease, also had agglutinating activity [8], it was found that cells from which one strain of myxovirus had eluted were still agglutinable by certain other strains, implying that the amount or destructibility of the enzyme-susceptible receptors required for binding and haemagglutination differed from strain to strain.

In the course of the studies on the virus-cell interactions, Burnet and his colleagues [8] furnished strong evidence in support of Hirst's view when they found that pretreatment of erythrocytes or host cells with a culture filtrate from certain bacteria, e.g. *Vibrio cholerae*, abolished their properties to agglutinate or adsorb influenza virus. This observation was correlated with the earlier reports of Friedenrich [3] and Thomsen [2] that erythrocytes became panagglutinable after treatment with culture filtrates of bacteria. By 1947 Burnet and his coworkers had established

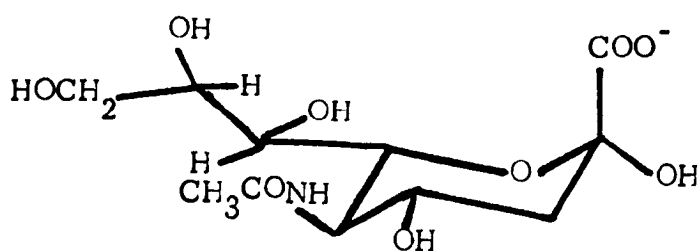
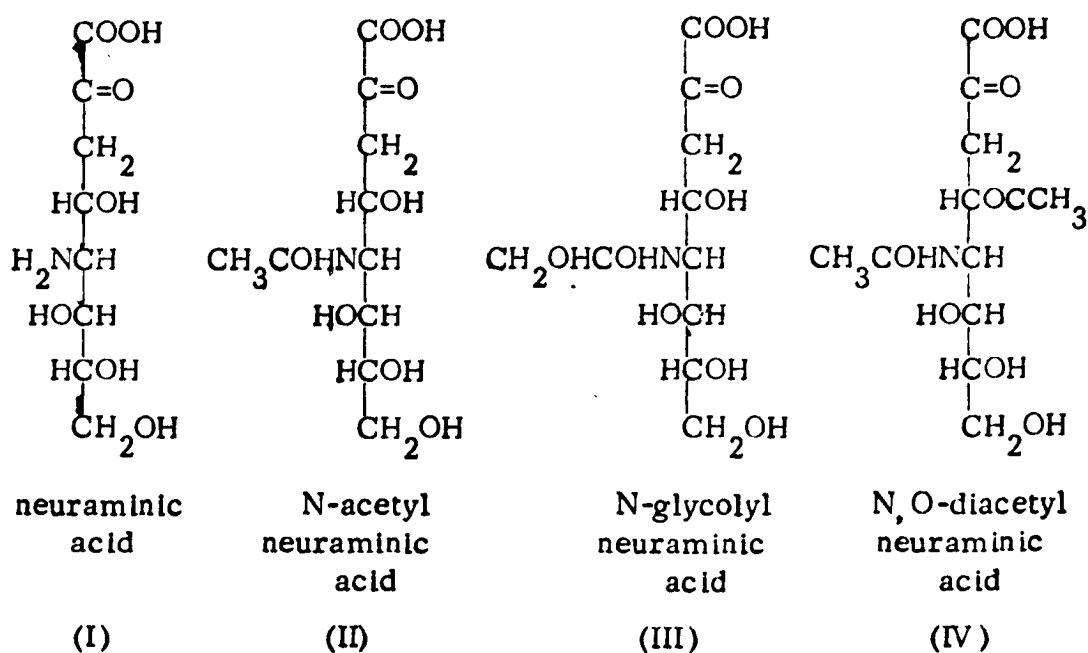
that the carbohydrate moiety of erythrocyte surfaces played a role in haemagglutination reactions and that this process could be inhibited by a number of glycoproteins [9, 10, 11]. They found that the carbohydrate receptor sites on the erythrocyte surfaces and on the glycoproteins which inhibited haemagglutination could be removed or inactivated by treatment with an enzyme present in the culture filtrate of Vibrio cholerae [12] or other bacteria [13], and since this enzyme destroyed the receptor sites for influenza viruses on the surface of erythrocytes it was termed 'receptor-destroying enzyme' [12].

Following the observation that influenza viral infections were focussed on the respiratory tract, the lining of which contains mucinous substances [14], it became necessary to conduct biochemical studies on the interaction between influenza viruses and mucins [9, 15, 16, 17]. Thus, by associating a particular substrate with an enzymic function, these studies further substantiated the enzymic nature of erythrocyte agglutination by virus. They also showed that the phenomena observed on treatment of cells with either bacterial filtrates or viral particles were due to one and the same enzyme found in both viruses and bacteria.

More information regarding the chemical nature of the substrate and the enzymic destruction of haemagglutination receptors came from further experiments in which (a) the product of the interaction between the influenza virus enzyme and ovomucin was characterised as an oligosaccharide containing one or more N-acetyl hexosamine residues [9, 18], (b) the viral enzyme and the receptor destroying enzyme from bacteria were classified as glycosidases [19, 24], (c) the product of the interaction of the receptor destroying enzyme with mucoproteins was isolated in crystalline form and identified as N-acetyl neuraminic acid, a sialic acid [20], and (d) the enzyme was recognised as an α -O glycosidase [21, 23].

According to the nomenclature suggested by Blix et al. [24], sialic acid is the group name for acylated derivatives of a nine-carbon sugar, with an amino group in the molecule, called neuraminic acid (Fig. 12).

Neuraminic acid never occurs in nature unsubstituted; usually it is found in its N-acetylated form, or as the N-glycolyl derivative or as various disubstituted derivatives (e.g. N,O-diacetyl). N-acetylneuraminic acid and probably all other sialic acids occur in the pyranose form and have the 1C conformation.



N-acetylneuraminic acid in pyranose form
(V)

Fig.1.2 Structures of some sialic acids

Heimer and Meyer [25] suggested that the name 'sialidase' be employed generally for the responsible enzyme whenever the product of the enzymatic action could be identified as sialic acid; since the destruction of receptor sites on mucoids capable of inhibiting Influenza virus haemagglutination can be mediated by enzymes other than sialidase, the general term 'receptor destroying enzyme' was retained to describe those cases where destruction of biological activity alone was determined. A year later, Gottschalk [26] suggested the use of an alternative term 'neuraminidase' which has gained fairly wide acceptance. The enzyme was defined as the specific α -glycosidase which catalyses the hydrolytic cleavage of the α -ketosidic linkage joining the potential keto group of a terminal N-acetylated neuraminic acid to an adjacent sugar in a disaccharide, oligosaccharide or polysaccharide [25, 26]. Figure 1.3 shows the action of neuraminidase on the glycosides of N-acyl neuraminic acids.

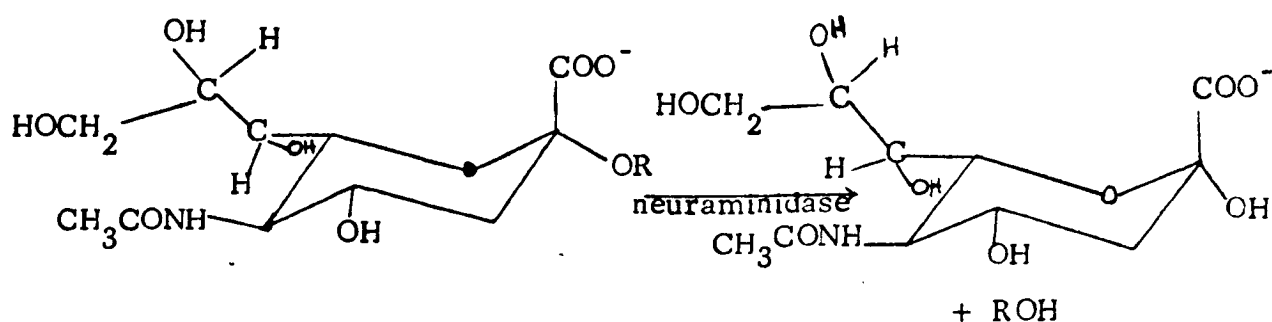


Fig. 1.3 The hydrolysis of an α -ketoside of sialic (N-acetyl neuraminic) acid by neuraminidase

R = monosaccharides, oligosaccharides, glycoproteins, glycolipids, aliphatic or aromatic alcohols.

Today the enzyme is listed by the International Union of Biochemistry under the systematic name 'Mucopolysaccharide (or glycoprotein) N-acetylneuraminyldiolase EC 3.2.1.18' according to Enzyme Nomenclature, 1965, [28], while retaining 'neuraminidase' as the recommended trivial name. The name 'sialidase' as originally proposed

by Helmer and Meyer [25] is preferred by many investigators who feel that this name is in better agreement with the presently proposed nomenclature of sialic acids [24] and that 'sialidase' is the more correct term because neuraminic acid (implied in the alternative term 'neuraminidase') is neither the product of the enzymatic action nor stable enough to exist per se in nature. Nevertheless, ^{the} term 'neuraminidase' appears to have a more universal use as is clearly attested by the titles and texts of many papers and reports (including this thesis).

1.2 Occurrence and Distribution of Neuraminidases

Neuraminidases comprise a group of rather diverse origin. They have been found in viruses, bacteria, protozoa and vertebrates. Neither neuraminidase nor sialic acid has so far been detected in plants.

1.2.1 Neuraminidase in viruses

As mentioned above, neuraminidase was the first enzyme to be found in a virus [6] and indeed was for a long time after its discovery the only virus specific enzyme known to be an integral part of myxoviruses ; it is only in the last few years that other enzymes, e.g. RNA- and DNA-polymerases, endo- and exo-nucleases and phosphohydrolases have also been associated with the virus particle.

Occurrence of neuraminidase in viruses is restricted to the ortho-myxovirus and paramyxovirus groups and not in other classes of virus. Indeed, myxoviruses were originally defined as those viruses 'having special affinity for mucins' [29], reflecting the presence of neuraminidase on their surfaces. From electron microscopic examinations, it has been suggested that viral neuraminidases are located on the surface of the virus where they form a significant feature of the surface morphology of the virus [30]. For parainfluenza viruses, the haemagglutinin and neuraminidase activities appear to reside on a single glycoprotein, although in the case of the influenza viruses the activities reside in different glycoproteins [31] (see Fig.1.4).

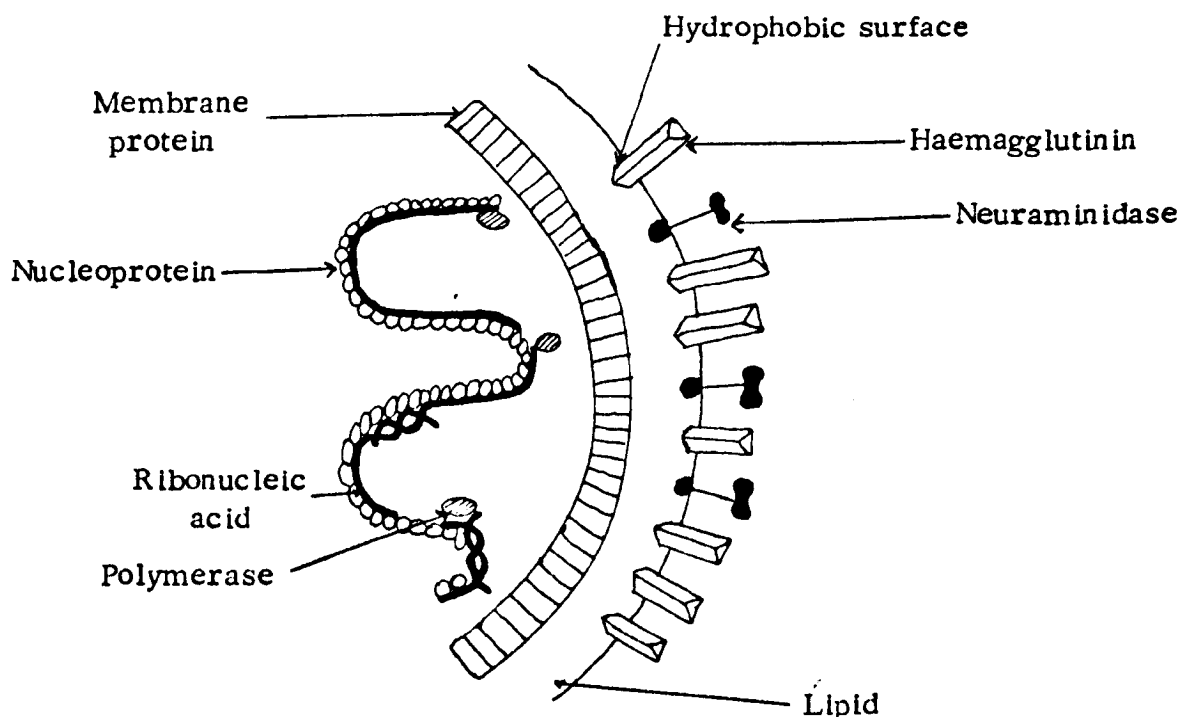


Fig.1.4 Diagrammatic representation of a segment of an influenza virus [30]

Since the synthesis of this enzyme is directed by the viral genome, neuraminidase from different viral strains have different antigenic, kinetic and physicochemical properties; also there is a several-fold difference in the amount of neuraminidase incorporated into a virus [32]. Depending on the virus strain and conditions of detection and isolation of the enzyme, neuraminidase generally represents 5 to 10% of the total viral proteins [30].

Table 1 summarises the occurrence of neuraminidases in myxoviruses, essentially as listed in recent reviews on neuraminidases [33, 34] except that influenza C has been omitted.

Table 1.1 Occurrence of neuraminidase in viruses

<u>Group</u>	<u>Type of virus</u>
Orthomyxoviruses	Human influenza, type A
	Avian, porcine, equine influenza
	Human influenza, type B
<hr/>	
Paramyxoviruses	Newcastle disease
	Mumps
	Parainfluenza, types 1, 2 and 3
<hr/>	

An earlier report that influenza C virus contains neuraminidase [35] has been revised, and it is now thought that influenza C has a receptor destroying enzyme which does not possess the enzymatic properties of neuraminidase but has an analogous effect on its own receptor, enzyme and haemagglutinating activity [36]. Also measles, distemper and rinderpest viruses classified as paramyxoviruses have been found to contain no neuraminidase. There has been no report in the literature that neuraminidase occurs in parainfluenza type 4 virus.

It is interesting that viruses such as influenza which contain neuraminidase lack sialic acid in their surface glycoproteins, presumably as a result of the viral neuraminidase activity [37] while viruses such as Sindbis or rabies in which no neuraminidase has been detected contain sialic acid residues at their surfaces.

1.2.2 Neuraminidase in protozoa and bacteria

The history of bacterial neuraminidases dates from the time of Burnet's concept of the existence of a receptor destroying enzyme [8, 12] which was first detected in Clostridium perfringens [13] and later in Vibrio cholerae [12]. The discovery of neuraminidases in bacteria and protozoa then entered a logarithmic phase and today more than 60 neuraminidases from different bacteria and protozoa have been described. Müller [39] has reviewed the occurrence of neuraminidases as reported in bacteria and protozoa in the period 1947-1974.

Bacterial neuraminidases are either cell-bound or are excreted into culture filtrates. For most bacteria the production of neuraminidase can be greatly enhanced by induction with free or bound sialic acid. [38, 40]. Recently one thousand microorganisms were screened for the production of neuraminidase, using colominic acid as the sole source of carbon in the culture media [41]. Pathogenic microorganisms such as *Corynebacteria*, *Bacteroidaceae*, *Clostridia*, *Vibrios*, *Streptococci*, *Pasteurellae*, *Klebsiella aerogenes*, *Erysipelothrix insidiosa*, *Mycoplasma gallisepticum* and the protozoa *Trichomonas foetus* tend to produce more neuraminidase than non-pathogens. Indeed, some authors have correlated the pathogenicity of these microorganisms with neuraminidase activity [39]. A few non-pathogenic bacteria, e.g. *Lactobacillus bifidus* [42] and *Streptomyces griseus* [43] have been found to produce neuraminidase. Microorganisms which possess sialic acid have weak or no neuraminidase and acylneuraminate-pyruvate lyase (NANA lyase, NANA aldolase EC.4.1.33) activities. Bacteria which are well established neuraminidase producers also possess NANA-lyase but their cells do not contain sialic acid. It is possible that strong neuraminidase-producers have evolved their ability to produce the enzyme as their adaptation to the inhabitation of sialic acid-containing substances, e.g. the respiratory and intestinal tracts [44].

1.2.3 Distribution of neuraminidases in vertebrates

Neuraminidase activity has been detected in different organs of birds and mammals [33, 34, 45]. The enzyme activity is high in those organs, e.g. liver, brain, kidney and mammary glands which are associated with a high metabolism of sialic acid and its derivatives [46]. Whereas vertebrate species all contain substrates for neuraminidase, viral and bacterial species which produce neuraminidase appear to contain no substrates for it. Most of the neuraminidase activity within calf brain has been found in grey matter where it is mainly associated with the synaptosome membrane [47]. In animal cells neuraminidase is bound to lysosomes, but has also been found associated with other organelles, e.g. microsomes and mitochondria [48]. In a number of tissues neuraminidase activity has been shown to vary with the age, physiological function and condition of the tissues concerned.

1.3 Importance of Neuraminidases

Sialic acids occur widely if not universally as terminal carbohydrate residues in glycoproteins, glycolipids and polysaccharides and also form a quantitatively important structural part of the cell surface. The participation of sialic acids in the functions of these substances has achieved widespread recognition through extensive studies in which the selective removal of sialic acid residues with neuraminidase has been associated with altered properties of the normal sialylated structures. The biological functions in which the involvement of sialic acid has been suggested include: cell-cell interactions, cellular transformations, blood clot formation, alteration of the half-life of circulating sialocompounds, neurotransmission, transport, interaction of hormones with their target cells, immunological properties and reproduction. In addition to the metabolic significance of neuraminidase in these physiological phenomena, neuraminidase function has been implicated in pathogenesis and its therapeutic efficacy has been assessed. It has also been employed as a tool in various structural investigations.

1.3.1 Some effects of neuraminidase on cell surface properties

Sialic acids may have direct or indirect biological roles as has recently been emphasised in a number of reviews on the chemistry and biology of cell surfaces [49, 50, 51, 52, 53]. The indirect roles of sialic acid attributed to the masking of cell surface antigens and other receptors can be ascertained by the enhanced antigenicity or other interactions created by the removal of sialic acid residues from the cell surface [54, 55].

The direct roles of sialic acid result either from the chemical properties of the carboxylic group or from the rigidity of the membrane due to the chemical properties imparted by the charged groups. In these respects sialic acid may be involved:

- (a) in nonspecific repulsion of cells or macromolecules by virtue of its strong negative charge [56, 57],

(b) as a receptor for:

- (i) various lectins [58, 59]
- (ii) virus particles [60, 61, 62]
- (iii) mycoplasmas [63]
- (iv) antibodies to cell surface antigens [64, 65]
- (v) hormones [66, 67]
- (vi) circulating glycoproteins (except transferrin) [68]
- (vii) tetanus toxin [69],

(c) in cell-cell interactions:

- (i) attachment of cells to inactive supports (adhesion) [70]
- (ii) attachment of cells to each other (aggregation) [71, 72, 73]
- (iii) attachment of cells to antibodies, other proteins, charged polymers or blood components [74, 75],

(d) in maintaining cell surface rigidity [56, 76] ,

(e) in physiological events:

- (i) stimulation of phagocytosis [57, 77]
- (ii) transport of ions, amino acids, proteins [78, 79, 80]
- (iii) synaptic transmission and brain excitability [81, 82]
- (iv) life-time of cells and sialo compounds [83, 84]
- (v) reproduction [85, 86, 87],

(f) in normal and malignant or transformed cells [88, 89, 90]

Although the exact role of cell surface sialic acid is not yet clear in the cellular functions mentioned above, it is interesting to note that its removal from the cell surface with neuraminidase is associated with a variety of altered properties of these cells (Table 2).

It is also interesting that the total cell surface sialic acid changes throughout the cell cycle : sialic acid increases before cell division and decreases after the onset of mitosis [99, 100, 101]. Further, old erythrocytes have less surface sialic acid than young cells and it is now established that sialic acid-deficient cells are recognised and removed from circulation.

The possibility that cellular neuraminidase activity is involved in these events, perhaps as a controlling mechanism for growth and metabolism, is being explored in many laboratories.

Table 2 Some effects of neuraminidase on cell surface properties

1. Reduces electrophoretic mobility (and negative charge) of a variety of cells [49, 52, 53].
2. Changes homing properties of lymphocytes [91].
3. Increases deformability of cultured cells [56].
4. Increases rosette-forming ability [92].
5. Decreases pinocytic behaviour of the cell surface [52, 53].
6. Increases susceptibility to phagocytosis of cells [57].
7. Alters transport of K^+ ions across cell membrane [78].
8. Decreases accumulation of α -aminoisobutyric acid in cells [79].
9. Alters viral-induced haemagglutination [52].
10. Reduces adrenal response to ACTH* [66, 67].
11. Increases adrenal response to cholera enterotoxin [92].
12. Shortens erythrocyte lifetime [83, 84].
13. Reduces penetration of ova by sperms [93].
14. Suppresses pregnancy [94].
15. Increases immunogenicity of various cells [52, 53, 95].
16. Increases cytolysis of cells by the immune components [96].
17. Increases platelet aggregation [75].
18. Detaches cells from glass substrate [70].
19. Delays mouse skin graft rejection and skin hypersensitivity [97].
20. Regresses mammary carcinoma [52, 53].
21. Inhibits release of proteins [80].
22. Abolishes synaptic transmission in synaptic structure [81].
23. Generates cell-mediated cytotoxicity [52, 53].
24. Reduces response of smooth muscle tissue to serotonin [52, 53].
25. Destroys virus receptors [7].
26. Stimulates cell division in cells at confluence [98].
27. Reduces precipitation of cells with protamine sulphate or hexadimethrine bromide [74].

*
ACTH - Adrenocorticotrophic hormone

1.3.2 Some effects of neuraminidase on glycoproteins

Treatment of various glycoproteins and other sialic acid-containing substances with neuraminidase has been shown to destroy or modify their biological activities and change such physicochemical properties as electrophoretic mobility, heat- and pH-stability, solubility, and microheterogeneity.

1.3.2.1 Biological activity of glycoproteins

The effects of experimental removal of sialic acid from biologically important sialic acid-containing substances with microbial neuraminidases have been reviewed in detail by several authors [49, 52, 102, 103, 104].

1.3.2.2 Stability and kinetic properties of glycoproteins

Results from several investigations have led to the thought that sialic acid may influence the stability and kinetic properties of glycoproteins. Treatment of human Tamm and Horsfall urinary glycoprotein / ^{with neuraminidase} resulted in an increase in the number of low pK tyrosine groups and in an increase in the number of titratable arginine groups [105], possibly suggesting that the carboxyl groups of sialic acid and the hydroxyl groups of tyrosine or arginine are directly hydrogen-bonded together. Some glycoproteins show considerable resistance to enzymatic proteolysis unless their sialic acid is first removed, e.g. with neuraminidase [106, 107] and, as mentioned earlier, such removal of terminal sialic acid has been shown to result in rapid elimination of the glycoprotein from plasma and uptake by the liver cells [84].

The stability of γ -glutamyltranspeptidase in acid was increased by neuraminidase treatment [108] and treatment of acid phosphatase with neuraminidase increased its optimum pH, substrate affinity and sensitivity to inhibition [109]. Enzymatic removal of sialic acid from choline esterase gives rise to a mixture of kinetically distinguishable forms of enzymes, one of which was specific for acetylcholine only [110]. Sialic acid has also been suggested as the component

responsible for the low isoelectric point, high electrophoretic mobility and chromatographic, electrophoretic and immunologic heterogeneity. Often the enzymatic removal of sialic acid will reduce or remove the heterogeneity, and in the case of isoenzymes the presence of subunits can be distinguished from the occurrence of different charges in different forms of the enzyme [111].

1.3.3 Neuraminidase in metabolism and physiology

The important roles of sialic acid, most of which have been suggested above, must inevitably apply to neuraminidase as well. In this section, therefore, the significance of neuraminidase upon the chemical and physiological state of its endogenous substrates and upon the functional aspects of other biological activities, e.g. cell-cell interactions, growth and development of malignancy, reproduction, hormonal, enzymatic and immunological activity, and neurotransmission will be discussed only briefly. The turnover of glycoproteins and their physiological functions have been discussed [112, 113]. The in vivo functions of neuraminidase as well as the effects of activators and inhibitors on its activity must assume a central role in the metabolism of these substances.

Various effects of neuraminidase upon different hormones have been described; for instance it 'inhibits' insulin secretion [114] has insulin-like stimulating action [115] and inactivates some of the hormones involved in reproduction of chorionic gonadotropin (HCG) and follicle-stimulating hormone (FSH) [116]. Removal of sialic acid groups from cell surfaces by neuraminidase suppresses collagen synthesis [117], inhibits the release of proteins from cells and alters the K^+ and Na^+ flux [118]. It is interesting that the (K^+, Na^+) -activated adenosine triphosphatase in plasma membranes contains sialic acid [119].

Evidence has accumulated favouring the role of neuraminidase in regulating the catabolism of a wide range of sialic acid-containing substances, in cells, glycoproteins and polysaccharides. Exposure of terminal non-reducing galactosyl residues by removal of sialic acid provides a means by which the liver recognises and removes the defective

molecule from circulation [84]. This may be their normal catabolic pathway. It is interesting that substances such as lysozyme and albumin which are not normally cleared from circulation by rat liver are cleared if covalently linked to desialysed fetuin [120].

Neuraminidase may be involved in the control of immune processes, acting to unmask antigenic determinants on cells or other substances by removing sialic acid residues and increasing immunogenicity [95]. Neuraminidase has also been shown to increase the non-specific fixation of immunoglobulins to erythrocytes [121] and, together with its enhancement of cytolysis of treated cells by the immune components [96], this may be another mechanism for the destruction of defective molecules. As well as influencing the immunological apparatus, the principle of masking by sialic acid may well be at work in cell-cell and cell-glycoprotein interactions, in which events neuraminidase may be instrumental in their regulation.

Essentially all lysosomal hydrolytic enzymes of rat liver and kidney are glycoproteins and some contain sialic acid which is responsible for their solubility, acidity and high electronegative charge ; the acidic, more soluble forms of these enzymes can be converted to the basic forms by treatment with neuraminidase [122], but the biological significance of such enzyme multiple forms is still unclear. However, since these forms differ in solubility, pH stability, pH optimum and charge, they would be expected to possess different hydrolytic activities under physiological conditions. The inhibition of alkaline phosphatase [123] by sialic acid is perhaps another point of metabolic regulation effected by neuraminidase. The induction of vertebrate cellular neuraminidase by its substrates is not documented, but it is well known in bacterial neuraminidases [41, 42]. However, when investigating the appearance of neuraminidase activity in the course of vertebrate development, Carubelli [124] obtained results indicating that vertebrate neuraminidase, in analogy to bacterial neuraminidases might also be adaptive enzymes. Carubelli and Griffin [125] later

observed elevated neuraminidase activity in HeLa cells grown in the presence of hydrocortisone. Although this rise in activity is obviously not due to specific enzyme induction by substrates or related substances, the finding indicated that neuraminidase production in vertebrate tissues, like that in bacterial systems, may be under regulatory control.

Several investigators have reported that neuraminidase content in brain tissues is often related to the amount of its endogenous substrates [126] and that its activity on brain gangliosides may be influenced by ionic strength [127]. It has also been observed that intoxication in rats leads to high levels of lysosomal neuraminidase and decreased Golgi neuraminidase [128], while hypercapnia and the associated respiratory acidosis is believed to be responsible for the activation of brain glycosidases including neuraminidases [129]. The metabolic diseases discussed below also implicate neuraminidase in metabolic functions.

1.3.4 Neuraminidase and disease

1.3.4.1 Metabolic diseases

In a few cases the activity or lack of activity of neuraminidase has been thought to directly or indirectly influence the establishment of disease conditions in which the amounts of neuraminidase susceptible substrates are accumulated or are deficient in abnormal proportions. In other instances, however, the abnormal levels of the enzyme or its substrates have not been shown to be parallel.

Deficiency of various lysosomal hydrolases, including neuraminidase, have been associated with mucopolidoses, congenital disorders deriving in abnormal storage of glycoproteins and/or glycolipids [129]. Recently another form of mucopolidosis in fibroblasts was shown to be associated with increased sialic acid and deficiency of neuraminidase, but no other lysosomal hydrolase [130]. Reviewing the various forms of sphingolipidoses caused by the lack of specific hydrolytic enzymes, Brady [98] has suggested that in Tay-Sachs disease and in generalised gangliosidosis, neuraminidase may be present only in low amounts or may be absent. However, the more recent finding of similar levels of neuraminidase in brain tissues from normals and cases of Tay-Sachs disease [160] is not in keeping with such a view.

A low content of platelet sialic acid was observed in two cases of congenital thrombocytopenia with giant platelets [131], and can be correlated with the marked thrombocytopenia observed after injection of

neuraminidase into mice, rabbits and rats [132]. Further, persistent polyagglutinability involving all the blood cells of one patient has been shown to be related to a partial deficiency of sialic acid residues at the surface of the erythrocytes [133]. In these studies, however, the low levels of sialic acid were not correlated with the neuraminidase content nor were the high levels of sialic acid content of blood from patients with coxarthrosis [134]; it is possible that the altered levels of sialic acid result from changes in the activity of the enzymes, including neuraminidase, which regulate its metabolism.

Excretion of sialic acid in urine was found to be predominantly in the bound form in kwashiokor while normal children excreted this compound largely in the free form [135]. The abnormally viscous secretions in pancreas, lungs and intestinal tract of patients with cystic fibrosis are thought to arise from faulty glycoprotein metabolism. The salivary mucins of patients with this disease were found to be more resistant to exoglycosidases e.g. neuraminidase, of bacterial and glandular origin. A raised level of uromucoid (a substance related to the Tamm and Horsfall glycoprotein in urine) was reported in patients with calculous disease [136] but neither has the relationship between uromucoid and the process of calcification nor the correlation between neuraminidase activity and the high levels of this glycoprotein been reported. However, by virtue of their sialic acid content, glycoproteins are capable of binding calcium [137] and perhaps initiate the formulation of a calculus.

1.3.4.2 The role of neuraminidase in pathogenic microorganisms

All living organisms need to adapt themselves in the most advantageous fashion on their environment, and this is apparent in the successful adjustment of pathogenic viruses, protozoa and bacteria to their selected hosts. The ability to induce enzymes which enhance their adaptation potential contributes to this success. Neuraminidase is still probably the best example of such enzymes. Correlation between neuraminidase elaborated by pathogenic organisms and their life styles is

difficult, but it is of interest to note that neuraminidases from pathogens show higher affinity for their substrates and are produced in higher amounts than neuraminidases from non-pathogens [138].

The substrates of neuraminidase are widely distributed in animal tissues, secretions and excretions. Invading pathogens will encounter in their new environment in the host adequate numbers of inducers and substrates; the glycoproteins and cell membrane glycoconjugates, the protective mucus lining of respiratory systems, oral cavities, gastrointestinal tracts, urinary-genital, lachrymal and nervous systems - all will possess chemical groupings which will induce the synthesis of neuraminidase whose effect and that of other enzymes present is to remove sialic acid and subsequently other sugars which can serve as a ready source of energy for the parasites. Neuraminidase serves to modify the physicochemical characteristics of the protective mucus meant to hinder close approach of other cells, and by its adhesiveness to entrap invading microorganisms. Gottschalk [139] described the pathomechanism of influenza in the respiratory tract which, in molecular terms, proceeds by decreasing the viscosity of mucin. Neuraminidase may thus be a powerful weapon of these parasitic organisms to be used when they are threatened with solitary confinement in a coating of adhesive and protective host mucin. This way the host is exposed to more infection. Further, the mechanism of the host immune response is made less effective because:

- (i) Ig A, which is the major antibody response, occurs only after the early highly virulent phase of the disease, and is itself an inducer and substrate of neuraminidase. Although the desialylation of Ig A by neuraminidase affects neither the affinity nor the antibody specificity [140], its efficiency as a defence mechanism will depend upon the relative rates of proliferation of the pathogen.
- (ii) The other defence mechanism is perhaps the cell-mediated immune response involving the B and T type lymphocytes, both of which possess terminal sialic acids. When these sialic acid residues are removed by the neuraminidase of the pathogen, the lymphocyte's response to

mitogenic stimuli is enhanced and the presence of the pathogens will activate macrophages, leading to the release of hydrolytic lysosomal enzymes, of which lysozyme will damage lysozyme-sensitive microorganisms and neuraminidase will destroy the host's protective mucus lining and cell membranes, thus contributing to an inflammatory response [142].

In some cases a direct link has been established between the ability of these organisms to synthesise neuraminidase and their pathogenicity. The pathology of microbial neuraminidases has been reviewed; numerous sources of the enzyme were considered and diseases associated with neuraminidases were tabulated [39, 143]. However, some authors have not favoured such direct role for neuraminidase activity in pathogenic microorganisms. Thus White and Mellanby [144] were able to separate neuraminidase activity from the oedema-producing, haemorrhage-producing and lecithinase activities, and other workers [145] found no correlation between animal virulence of D. pneumoniae and its ability to produce neuraminidase. It is interesting, however, that Fedoseeva et al. [146] observed a direct relationship between the elevated levels of free sialic acid and the bronchopulmonary process in children with chronic pneumonia and asthma. Burton [147] reported that the neuraminidase of Clostridium perfringens was not toxic to mice whereas, in other reports, neuraminidase was thought to be the causative agent in the toxicity of the component produced by Clostridium perfringens in food poisoning [148]. Also the factor in the culture filtrates of Vibrio cholerae which inhibited the sodium pump of frog skin was shown to be different from the Vibrio cholerae neuraminidase [149].

1.3.4.3 The role of neuraminidase in viral infections

In the case of viral infections there have been a number of conflicting lines of evidence in support of different roles of viral neuraminidase in the replication of viruses. The original suggestion of Hirst [7] that neuraminidase assists in the penetration of the virus into the host cell by cleaving sialic acid residues from cell surfaces

has been doubted following observations that heat-inactivation [150] or proteolytic removal [151] or inhibition [152] of viral neuraminidase does not affect the infectivity of the virus, suggesting that neuraminidase is not essential in the early stages of influenza virus replication. In other reports, however, the inhibition of viral neuraminidase with 2-deoxy-2,3-dehydro-N-acetylneuraminic acid [153] or with 2-deoxy-2,3-dehydro-N-trifluoro-acetylneuraminic acid (Fig.1.5) [154] inhibited the replication of influenza viruses. It is possible that the attempts to exclude neuraminidase activity from the virus particle may not have been completely effective so that some residual neuraminidase activity remained functional. Other neuraminidase inhibitors such as phenylglyoxal or phenylglyoxamic acid have not been shown to inhibit viral replication.

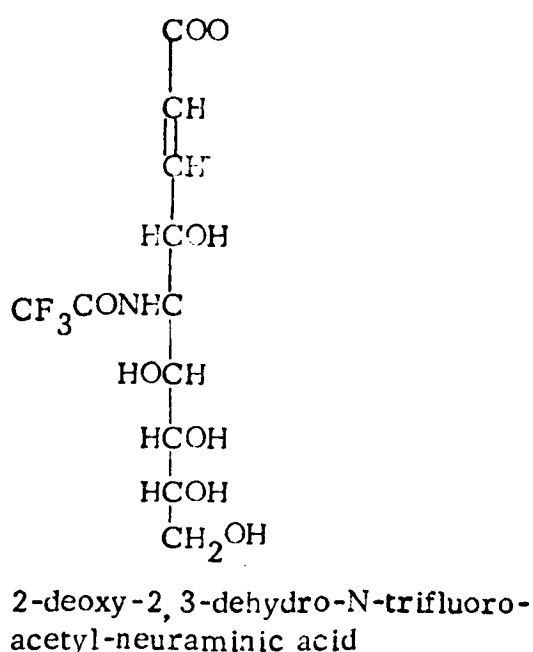
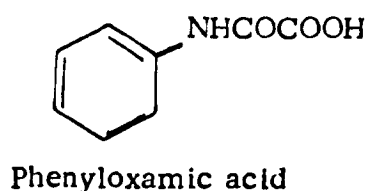
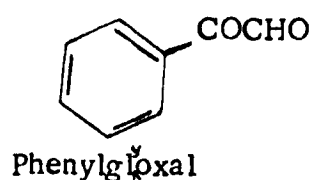


Fig.1.5 Inhibitors of viral neuraminidase [154]

The role of viral neuraminidase in binding myxoviruses to host surfaces was suggested in several reports, e.g. [155, 156], following experiments in which the removal of sialic acids from cell surfaces diminished or completely prevented the attachment

of viruses to cells. Later the function of sialic acid in such attachment was further confirmed using artificial lipoprotein membranes which bound myxoviruses but not rhinoviruses or herpes viruses [157]. Other observations, however, did not favour the idea that an enzyme-substrate interaction was responsible for the binding of viruses to cells. For instance, it had been noted earlier that neuraminidase-specific antibodies neither affected the agglutination of red cells by myxoviruses [158] nor the binding of myxoviruses to erythrocytes [159]; moreover, the neuraminidase liberated and purified from the virus failed to adsorb to red cells [159]. However, these studies are not necessarily conclusive, especially in view of the existence of variations in both the specificity and viral content of neuraminidases in even antigenically identical virus recombinants [32].

The role of neuraminidase in the release of progeny virus from the cell surface has also been suggested [161] mainly from observations on the effects of antibodies to viral neuraminidase on viral replication; but Kendal and Madeley [162] and Becht *et al.* [163] have concluded that the observed effects of antibodies to neuraminidase were not due to their interference with neuraminidase enzymic activity but rather arose from binding virions to each other and to infected cells. The viral enzyme is therefore not responsible for the release of myxoviruses from the cell surface. In addition, the time of maximal neuraminidase production in an infected cell is long before the release of the virus [164].

Nevertheless, there is mounting evidence suggesting correlation between immunity to viral infection and the presence of antineuraminidase activity. Antibodies against purified viral neuraminidase have protected mice [165], cattle [166] and man [167] against influenza. Also the inactivation of neuraminidase to destroy the receptors for the virus protected mice from influenza [168], and in similar experiments

Cairns [169] obtained a moderate degree of protection against intracerebral infection of a neurotropic variant of influenza ^{following} strains/ inoculation of large amounts of neuraminidase. Interferon, a cellular antiviral substance, was shown to suppress the production of neuraminidase in the growth of influenza virus in chick embryo cell cultures [170].

One so far unquestioned role of neuraminidase in the replication of viruses is the part it plays in viral assembly by preventing aggregation. It is known that viruses which contain neuraminidase possess no sialic acid residues, presumably due to the function of viral neuraminidase [37]. If sialic acid residues were incorporated in virions containing neuraminidase, they would serve as receptors for the viral haemagglutinin and result in aggregation and consequent low yield of infectious virus. Thus neuraminidase protects the virus from its own agglutinin.

1.3.5 Direct applications of neuraminidases

Since their discovery, neuraminidases have been used a great deal in research to study their effects on the removal of sialic acid residues from cell surfaces and various other biologically important sialic acid-containing compounds, e.g. enzymes, hormones, glycolipids, etc., many of which were discussed above. Most of these studies have been conducted with microbial neuraminidases since the viral enzyme is often unavailable in sufficient amounts. The alternative chemical methods of removing sialic acid from its natural partners, e.g. acid hydrolysis, methanolysis, often are not specific and can be incompatible with most properties of the substances under study.

In addition to their value in assessing the content, the biological function and the nature of the linkage of sialic acid in glycoproteins etc., neuraminidases have been used to remove sialic acids from glycoproteins and thus correct for the amide nitrogen arising out of sialic acids, during acid hydrolysis of glycoproteins [171].

The differences in the properties of neuraminidases from different sources have been proposed as a basis for the classification of viruses [172] and vibrios [138].

Some viruses have been purified by virtue of their neuraminidase content. Thus influenza viruses can be concentrated and purified by adsorbing on to red blood cells at 4° C, raising the temperature to 37° C after the cells have been washed free of impurities, and recovering the purified virus by centrifugation [173]. Similarly the neuraminidase inhibitor, p-nitrophenyloxamic acid, was used to purify neuraminidase-containing viruses [174] and recently immobilised neuraminidase was used to purify interferon [175]. Neuraminidase has also been used as a means of assaying the biological activity of Interferon [176].

The potential of neuraminidase in the therapy of viral infections has been assessed by a number of investigators. Protection against viral infections by antibodies to neuraminidase [165-167] or by treatment with neuraminidase itself [168, 169] was mentioned above, and although these methods of inhibiting viral replication have not so far gained clinical use, further studies may make it possible. Treatment of normal or malignant cells with neuraminidase increases their immunogenicity and imparts an increased antitumour activity [52, 53, 95, 177]. The induction of an immune response towards tumour-specific antigens during natural antibody-initiated clearance of neuraminidase-treated cells is thought to be the mechanism underlying the therapeutic effect observed following inoculation of tumour-bearing rodents with neuraminidase-treated cells [177], but whether this method of immunotherapy can be applied to the clinical management of human cancer is yet to be established.

1.3.6 Interpretations of the experimental use of neuraminidases

There have been many conflicting reports relating to the interpretation of the results obtained, especially following the removal of sialic acid residues from whole cells. Treatment of

cells with neuraminidase is not considered to be too harmful since, in the case of red cells, the properties of osmotic fragility, autohaemolysis at 37°C , K^{+} retention and pyruvate kinase activity of untreated cells are maintained [52]. However, it has been shown that neuraminidase can enter [178] or bind to [179] cells, both of which phenomena could lead to various effects, (e.g. masking of adjacent functional groups, breakdown of metabolites, etc.), and thus cause aberrant behaviour not related to the specific group or function under study. Recently Parker *et al.* have described the use of immobilised neuraminidase which promises to alleviate such technical problems [188]. The interpretation of the effects of neuraminidase on cells etc. and their function is further complicated by the considerations that different cells contain different types of glycosidic linkages in various proportions which are cleaved to different extents by neuraminidases from different sources [33, 34, 53]. This will be discussed in the next chapter. Although the particular biological function may be related to a specific glycosidic linkage, the removal of sialic acid may be non-specific and some of the sialic acid residues may be resistant or poorly hydrolysed either by virtue of the α -ketosidic linkage or because of steric hindrance. Often the substances on which the effect of neuraminidase is studied may have been originally obtained from tissues also known to contain neuraminidases, and the cases in which experimental addition of neuraminidase were seen to have no effect may well be due to the previous function of tissue neuraminidase in vivo, after death or during the preparation of the substance under investigation.

Lastly, the definitive information obtained by using neuraminidase to modify cell membranes, glycoproteins, etc. must depend on having a rigorously purified enzyme or otherwise establishing that the effect is due to the enzyme under investigation. Observations by Kraemer [180] that commercial neuraminidase contained cytotoxic, haemolytic and phospholipase activities demonstrated the necessity to further purify the enzyme before its use in establishing the biological roles of

sialic acid. Another serious contaminant of the commercial enzyme is proteolytic activity [181]. Considering that most of the neuraminidase preparations employed in these studies were never evaluated for contaminants and were used without purification beyond commercial quality, the results obtained are necessarily difficult to interpret. For instance, loss of homing properties similar to that observed on treatment of lymphocytes with neuraminidase has been observed after treating the cells with trypsin [182], and cell surface trypsin-mediated proteolysis has been shown to modify cell growth [183], experimental pulmonary metastasis [184], cell agglutinability [185] and aggregation/adhesion [186].

1.4 Aim of the Project

Clarification of these conflicting results and the definition of the exact role of neuraminidase will inevitably follow the complete characterisation of the enzyme.

Neuraminidase has for the most part, since its discovery, been studied by virologists interested in the virus or by cell-biologists interested in assessing the function of sialic acids through their removal from cell surfaces or other sialic acid-containing cellular metabolites, and by chemists interested in it as a tool in structural investigations. Bacterial neuraminidases have been, and are presently, regarded by some as model substance of the viral enzymes studied mainly because of lack of sufficient viral material. So, although it is over thirty years since the first neuraminidase was studied as an enzymic entity [12], the detailed chemistry of the structure and function of these enzymes is still obscure. However, a great deal of work has been done on the enzymic action of neuraminidase on different natural and synthetic substrates as well as its inhibition by various chemically defined inhibitors. The kinetic and physical properties described for neuraminidases from different sources have revealed certain important features of these enzymes.

Having introduced the biological importance of neuraminidase (although for the most part yet uncertain) , it is difficult to exaggerate the necessity for accumulation of enough knowledge about the enzyme's chemical properties in attempts to define more exactly its function in molecular terms. The greatest emphasis in the studies described below will therefore be on chemical properties of different neuraminidases with a view to understanding the way in which their functional properties are the phenomena that their structure must explain.

CHAPTER TWO

PROPERTIES OF NEURAMINIDASES

2.1 Introduction

The properties and differences of neuraminidase from different sources have been described in recent reviews [33, 34, 45, 102, 187]. Generally bacterial and vertebrate neuraminidases occur as single polypeptides with molecular weights in the range 50,000-100,000, although some bacterial neuraminidases with molecular weights below and above this range have been reported [43, 187, 189]. Viral neuraminidases possess multiple polypeptide chains, have molecular weights in the neighbourhood of 200,000 and their pH optima are closer to neutrality than the rather acidic pH optima generally found for bacterial and vertebrate neuraminidases. The occurrence of multiple forms of neuraminidase has been observed mainly from chromatographic indications of more than one peak of neuraminidase activity during purification of this enzyme from a number of bacteria [190, 191]. Whether this microheterogeneity of neuraminidase preparations was due to genuine genetically determined isoenzymes or to the aggregation and disaggregation of molecules, or to differences in conformational states, or to differences in subunit dissociation and recombination or to heterogeneity in ligand binding, or to the effects of contaminating proteases or simply to the formation of artefacts has not been established. The data presented by Tanenbaum and Sun [192] restricts the choice of hypotheses for the manifestation of isoneuraminidases [190] to those involving charge differences and indicate a lack of subunit structure for such multiple forms. In the cases in which the multiple forms of neuraminidase were reported, no affinity chromatographic separation step was involved; further, the specificities of the different forms of neuraminidase towards different sialic acid substrates were not investigated. The substrate specificity, kinetics, metal ion requirement or inhibition by different agents varies with the source of the enzyme and with the substrate used.

In natural substrates of neuraminidase (Figure 2.1), carbon 2 of sialic acid is either linked to carbon atom 3, 4 or 6 of galactose, carbon atom 6 of N-acetylgalactosamine or N-acetyl-D-glucosamine or to carbon atom 8 of a second sialic acid molecule [193].

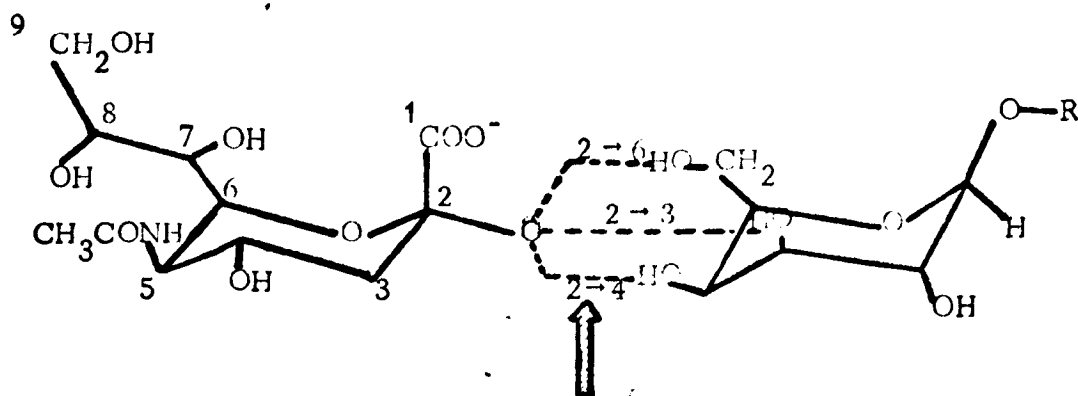


Figure 2.1 (arrow). Action of neuraminidase on the possible glycosidic linkages between sialic acid and the penultimate sugar in a neuraminidase substrate.

With few exceptions [33, 34, 45, 102, 187] bacterial and vertebrate neuraminidases cleave most α -ketosidic linkages (α -2 \rightarrow 3, α -2 \rightarrow 4, α -2 \rightarrow 6 and α -2 \rightarrow 8), although with differing ease, depending on the type of substrate, enzyme source and analytical conditions. In contrast, viral neuraminidases preferentially hydrolyse α -2 \rightarrow 3 linkages. The natural substrates of neuraminidase vary widely in size and contain varying amounts of releasable sialic acid. Table 2.1 lists some of the substrates more commonly used in assaying neuraminidase.

Neuraminidase activity is commonly determined by measuring the amount of sialic acid produced during the enzymatic hydrolysis of the appropriate substrate, usually by the thiobarbituric acid method [196]. Methods in which the released aglycone or the decrease in bound sialic is determined have also been described. Until today, one of the major setbacks in research involving neuraminidase has been the lack of a standard assay procedure by which all neuraminidases can be determined.

Table 2.1 Natural neuraminidase substrates *

Substrate	Substrate source	M. wt.	% by wt. NANA content†	Type of ketosidic link								N-substituent	Reference
				2 → 3	2 → 4	2 → 6	2 → 8						
Sialyllactose	Cow colostrum	633	49	+	-	+	-	-	-	-	acetyl	187	
Colominic acid	<u>E. coli</u>	ca. 5,000	90-100	-	-	-	-	-	-	400	acetyl	194	
α-Acid glycoprotein	Human plasma	44,100	12	+	+	+	+	+	+	-	acetyl	187	
Fetuin	Foetal calf serum	48,000	9	+	-	+	-	+	-	-	[acetyl(93%) glycolyl(7%)]	187	
Submaxillary mucin	Bovine submax gland	1.2x10 ⁶	21	+	n.d.	+	n.d.	+	n.d.	n.d.	[acetyl glycolyl O,N'-diacetyl]	187	
Collocabin mucoid	Edible birds' nest	2 x 10 ⁶	9-13	+	n.d.	n.d.	n.d.	+	n.d.	n.d.	acetyl diacetyl	187	
Di- and Tri-sialic gangliosides	Bovine brain	1,880	23-30	+	-	-	-	-	-	+	acetyl	195	

* + type of linkage indicated has been reported.

- type of linkage indicated is known to be absent

n.d. presence or absence of linkage has not been documented.

† NANA - N-acetylneuraminic acid.

As a result, it has often been difficult to compare or meaningfully interpret the observed properties of different neuraminidases. Thus, the amounts or properties determined may reflect the substrate specificity of the enzyme, which varies with the number of different linkages in the same substrate, or the limitation in the detection system, e.g. low sensitivity, interference, reproducibility or the differences in the purity of both the enzyme preparation and the substrates used.

Often the variations obtained when measuring different sialic acid may relate to the nature and amounts of the sialic acids involved. For instance, the differences in acylation have been thought to be responsible for the markedly different molar extinction coefficients exhibited by different sialic acids in the thiobarbituric acid test procedure [34].

Although in the last few years methods of increased sensitivity for the determination of neuraminidase activity have been described, e.g. by use of natural substrates with radioactively labelled sialic acid moiety [197] or aglycone [198] or by use of crystalline synthetic substrates of more defined structure [187] or by use of fluorimetric [199] or gas chromatographic [200] techniques or by use of coupled enzyme systems [201] the thiobarbituric acid method [196] of determining sialic is very widely used because of its relative sensitivity and convenience [202].

As well as finding a uniform, sensitive and specific assay for neuraminidase activity, current research on the enzyme has been concerned with its extensive purification from different origins, its application to molecular biological investigations and its chemical characterisation. Considering the wide range of sources of neuraminidase in vertebrates, protozoa, bacteria and viruses, it is not surprising that the methods that have been adapted in its purification are quite different [33, 34, 45, 102, 187].

Recently, affinity chromatographic procedures based on the interaction between the enzyme and either the inhibitor, e.g. N-(p-amino-phenyl)oxamic acid [174], 4-(Nitrophenyl)oxamic acid [204], N-acetyl-neuraminic acid [205] or the substrate, e.g. α_1 -acid glycoprotein [206],

fetuin [31, 207], colominic acid [208] have been added on to the conventional enzyme purification protocol as the last step.

In spite of the great wave of research dealing with neuraminidase, the number of laboratories privileged to prepare neuraminidase from their own bacterial culture is limited, and most use the commercial enzyme which is not only expensive but also contains various contaminating activities [180, 181, 207] and requires further purification prior to use. Neuraminidases are costly to prepare by existing methods, and it is a disadvantage that they depend on the use of pathogens which would impose health hazards and demand elaborate handling techniques. Certain non-pathogenic microorganisms have been found to produce neuraminidase [42, 43, 209] although in low amounts. However, non-pathogenic strains of Arthrobacter have recently been shown to be potent neuraminidase producers [210].

For the purposes of chemical characterisation of neuraminidases, the enzyme which was previously purified from a non-pathogenic strain of Streptomyces [43] was attractive. Its low molecular weight (32,000) and lack of subunit structures would be of particular advantage in interpreting chemical analyses and other studies in relative absence of intramolecular effects. Also, as a point of comparison, the molecular weights of several neuraminidases seem to be a multiple of the molecular weight of the Streptomyces enzyme (Table 2.2).

Table 2.2 Subunit relationship of some neuraminidases

<u>Source of neuraminidase</u>	<u>Molecular weight</u>	<u>Reference</u>
<u>Streptomyces griseus</u>	32,000	43
<u>Clostridium perfringens</u>	64,000	34
<u>Diplococcus pneumoniae</u>	69,000	34
<u>Vibrio cholerae</u>	90,000	34
Influenza B/Lee virus	240,000	187

Although the molecular weights of most other neuraminidases bear no such relationship to the Streptomyces enzyme, it would be of interest to compare chemical structural data of a number of purified neuraminidases. When this work was started, only the amino acid composition

of influenza A₂(X-7/F1) N2 neuraminidase had been published [211]; to date three more sets of amino acid composition data of different neuraminidases have been added [187, 210, 212]. Also presented along with this data has been the carbohydrate content of neuraminidases. It is reasonable to assume that the variations exhibited in the structural components of these enzymes reflect on the physical and functional properties of different neuraminidases.

In this chapter the isolation, purification and characterisation of a neuraminidase from Streptomyces griseus will be described. Chemical studies on the active sites of this and neuraminidases of other origin will be reported in Chapter 3.

2.2 Isolation, purification and characterisation of a neuraminidase from Streptomyces griseus

Experimental Procedure*

2.2.1 Growth of Streptomyces griseus

Streptomyces griseus MB395-A5 was grown at 27° C as previously described [43] except that α_1 -acid glycoprotein (or fetuin) was also included in the culture medium to increase the yield of neuraminidase by induction [40]. The medium used had the following composition:- glucose, 10.0 g; starch, 10.0 g; peptone, 7.5 g; meat extract 7.5 g; NaCl, 3.0 g; MgSO₄·7H₂O, 1.0 g; K₂HPO₄, 1.0 g; CuSO₄·5H₂O, 7.0 mg; FeSO₄·7H₂O, 1.0 mg; MnCl₂·4H₂O, 8.0 mg; ZnSO₄·7H₂O, 2.0 mg; α_1 -acid glycoprotein, 10.0 mg (or fetuin, 15.0 mg) and water, 1 litre. The media were sterilised by autoclaving at 15 psi for 30 minutes. For enzyme isolation, 20 litre flasks containing 17 litres of medium were inoculated with cells (100 ml) from a 70-hours-old culture that had been started from a loop of cells from an agar slant culture. After 72 hours of growth at 27° C, in a well-aerated culture medium, the culture fluid was harvested by filtration through a celite bed at 4° C; the filtrate containing the crude neuraminidase was used to purify the enzyme.

In studies to correlate the secretion of neuraminidase with the growth of Streptomyces griseus, one litre Erlenmeyer flasks, each containing 500 ml of the medium used above, were inoculated with a

* Unless otherwise stated, the sources of the special materials used are alphabetically listed in Appendix IV.

cell growth suspension (5 ml) from a 70-hours-old culture prepared as above. The flasks were shaken at 27^o C and 10 ml samples were aseptically extracted from the growth medium at different times and used to measure the cell growth (nephelometrically) and the corresponding amount of neuraminidase.

A fully grown culture of Streptomyces griseus was a yellowish, light brown colour and had a characteristic 'soily' smell. The cells formed white balls, the size of which depended on the aeration conditions. Faster aeration (bubbling or shaking) of the culture produced bigger balls; branched, thread-like structures were visible in cultures which were not aerated. However, the relationship between the degree of aeration and the amount of enzyme produced in the culture fluid was not studied. Microscopic examination of the growth culture revealed filamentous structure of branching mycelia (Fig. 2.2).

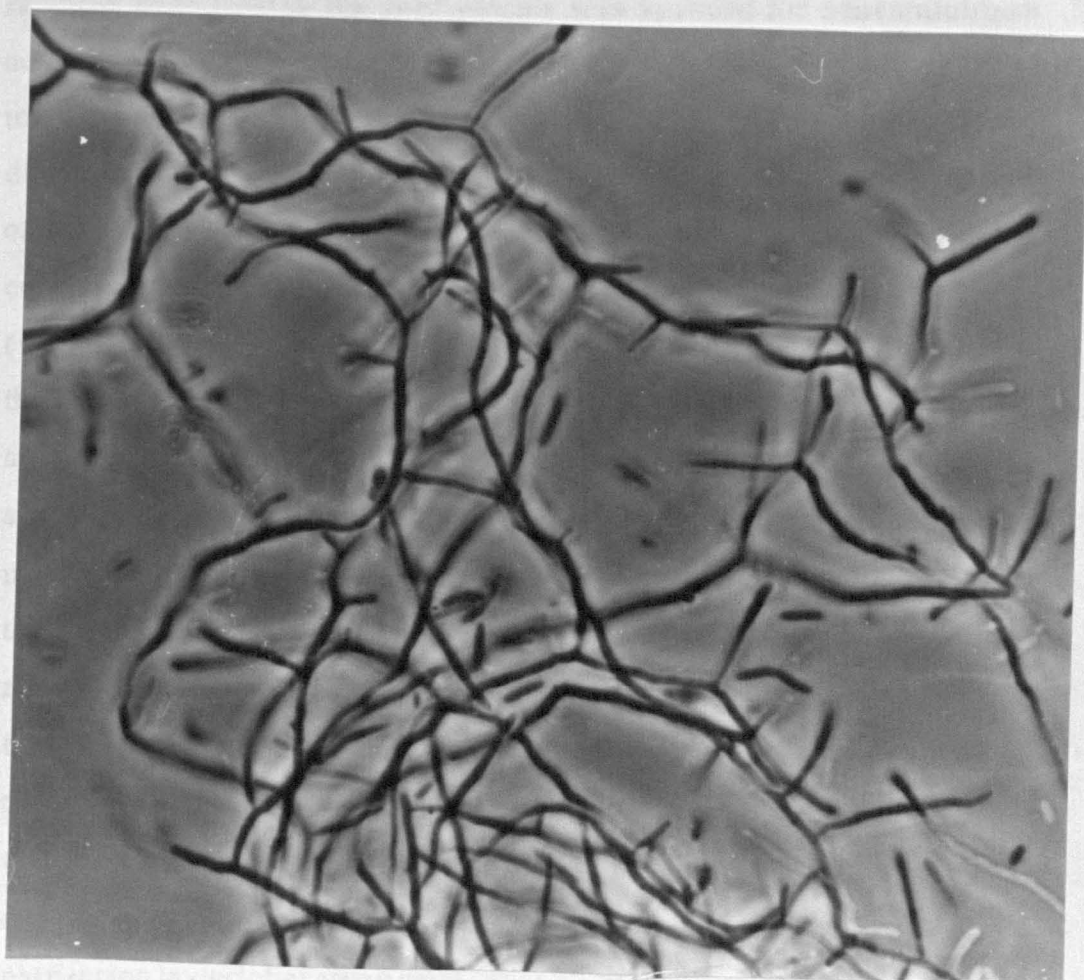


Fig. 2.2 Phase contrast photomicrograph of a 72-hours-old culture of Streptomyces griseus MB395-A5. Magnification: x 220.

2.2.2 Assay of neuraminidase in the culture fluid

The assay of neuraminidase activity by determining the sialic acid released enzymically from various substrates using the thiobarbituric acid method of Warren [196] is complicated when dealing with biological materials because of substances such as deoxyribose and related materials which yield an interfering colour product [196, 214]. In such samples the interfering colour product is often so intense that the colour changes due to small differences in sialic acid content can be missed altogether. In the present studies, a gel filtration column was introduced to separate the enzyme and other high molecular weight substances from the smaller sugar molecules, which interfere in the thiobarbituric acid test before the enzyme was assayed. The culture fluid (20 ml) to be assayed was passed through a Sephadex G.25 column (1.5 x 20 cm) previously equilibrated with 0.05 M sodium acetate buffer, pH 5.3, at 4° C and eluted with the same buffer. The fraction contained in the void volume was assayed for neuraminidase activity as follows: fetuin (prepared from foetal calf serum according to the procedure of Spiro [214]) or α_1 -acid glycoprotein were freshly dissolved in 0.05 M sodium acetate buffer, pH 5.3, at a concentration of 4 mg/ml and used as the substrates. The reaction mixtures contained the substrate (0.8 ml), buffer (0.2 ml) and the culture fluid (1.0 ml) and were incubated for 60 minutes at 37° C before conducting the thiobarbituric acid assay for the free sialic acid released by the neuraminidase in the culture fluid. Of the different ways employed to stop the enzyme reaction in the assays, e.g. by heating the incubation mixture to 100° C or adding protein precipitants, it has been found that the periodate reagent in 9 M phosphoric acid comprising the first reagent added in the thiobarbituric acid test is sufficient and convenient in stopping the enzyme reaction. In the blanks, the culture fluid or the substrate volumes in the tests were replaced by appropriate volumes of buffer (E_0 and S_0 respectively). The optical density readings at 549 nm and the absorption spectra of the resulting chromogens after extraction in cyclohexanone were determined and compared with those of crystalline N-acetyl neuraminic acid as the standard and those obtained for culture filtrates prior to the gel filtration step. The absorption

spectra, of which a typical example is shown in Fig. 2.3, were determined by individual readings at each wavelength.

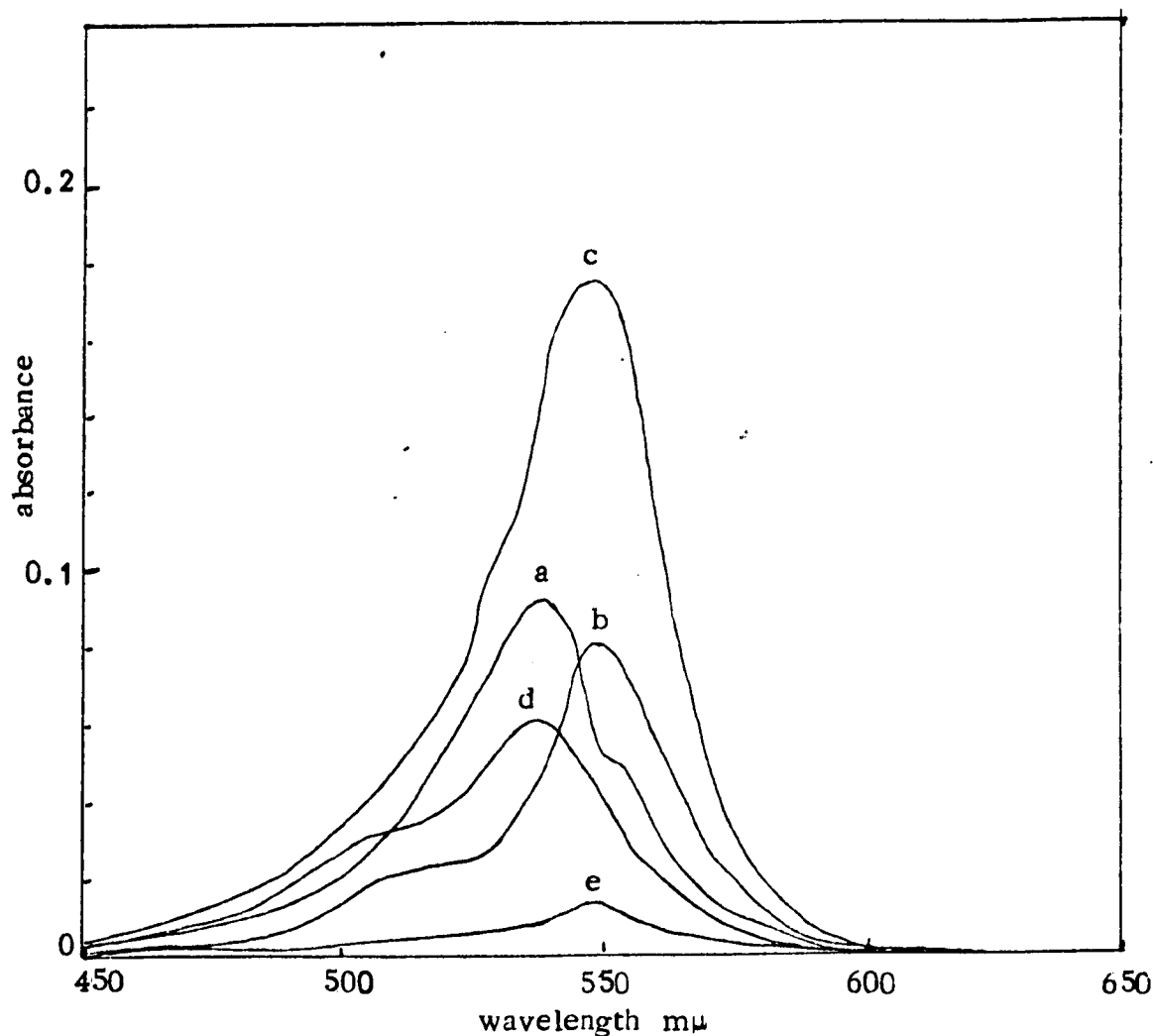


Figure 2.3 Absorption spectra of chromogens obtained in the thio-barbituric acid assay for sialic acid. Culture fluid assayed for Nase (a) prior to & (b) after the gel filtration step ; (c) N-acetylneuraminic acid ; (d) culture fluid without substrate (S_0); (e) substrate without culture fluid (E_0).

The protein concentration in the culture fluid was determined according to Lowry et al. [215]. Fig. 2.4 shows the production of neuraminidase during the growth of Streptomyces griseus MB395-A5.

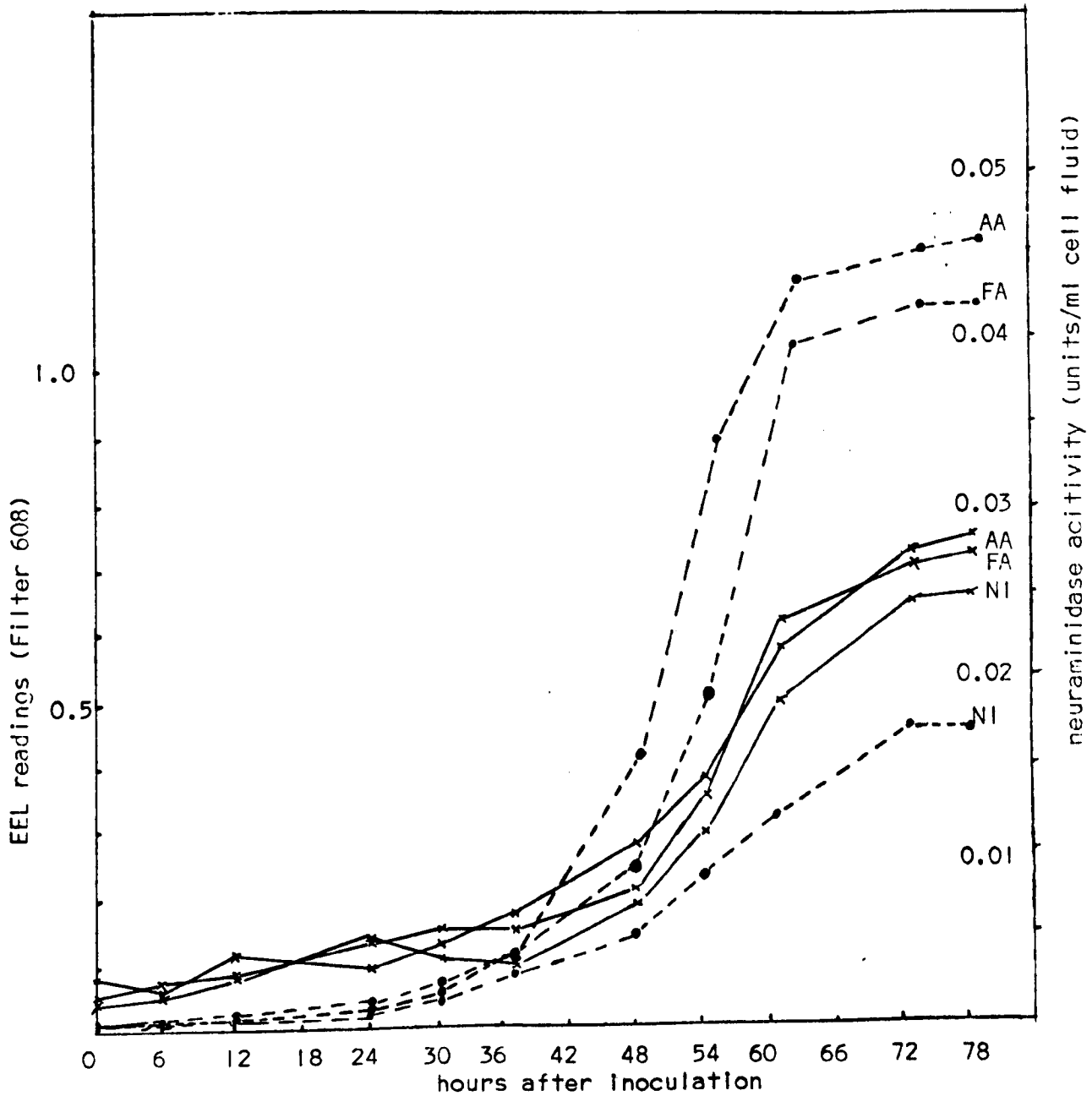


Figure 2.4 Production of neuraminidase during the growth of *Streptomyces griseus* MB395-A5.

(—x—x—) cell growth measured nephelometrically.
 (---•---) neuraminidase activity units per ml cell culture fluid. AA or FA induction by α_1 -acid glycoprotein or fetuin respectively.
 NI, no inducer added.

In only two sets of studies, the enzyme was induced by fetuin instead of α -acid glycoprotein. The fetuin-induced neuraminidase released sialic acid from fetuin much faster than did the α_1 -acid glycoprotein induced neuraminidase (Table 2.3).

Table 2.3

<u>Culture fluid</u>	<u>Units neuraminidase/ml using as substrate</u>	
	<u>α_1-acid glycoprotein</u>	<u>fetuin</u>
Without inducer	0.013	0.006
With α_1 -acid glycoprotein	0.054	0.032
With fetuin	0.029	0.041

2.2.3 Purification of neuraminidase from the culture filtrate

The crude enzyme from the culture filtrate of Streptomyces griseus was purified by a combination of precipitation (with ammonium sulphate), ion-exchange chromatography (on DEAE- and CM-celluloses), adsorption on hydroxylapatite and gel filtration on Sephadex G-100 [43]. All purification procedures were carried out at 4° C. Table 2.5 summarises the purification procedure. To assay fractions other than the culture fluid for neuraminidase activity, a 2 mg/ml solution of fetuin or α_1 -acid glycoprotein in 0.05 M sodium acetate buffer, pH 5.3, was used as the substrate (0.5 ml) and incubated with the enzyme fraction (0.5 ml) for 50 minutes before conducting the Warren assays for the released sialic acid [196]. Protein concentration was determined according to Lowry et al. [215].

Further purification was afforded by a final affinity chromatographic step using fetuin bound to cyanogen bromide-activated sepharose [31]. Sepharose 4B was activated with cyanogen bromide as described by March et al. [216] and the activated agarose was then coupled to fetuin as described in the method of Scheid and Choppin [31]. The resulting fetuin-Sepharose was stored in aqueous suspension with 0.02% sodium azide at 4° C until used. To assess the stability and effectiveness as adsorbent for neuraminidase of fetuin-sepharose in affinity chromatography, its sialic acid content was determined by the method of Warren [196] after release with neuraminidase incubation at 37° C for 2 hours or after hydrolysis in 0.125 NH_2SO_4 at 80° C for 1 hour [203]. These determinations were done on 1.0 ml settled predialysed fetuin-Sepharose; the neuraminidase incubations were done with 2 mg of

Clostridium perfringens neuraminidase, The sialic acid content determined, using crystalline N-acetyl neuraminic acid as the standard, is tabulated (Table 2.4).

Table 2.4 Sialic acid content of fetuin-sepharose

<u>Substrate</u>	<u>Total μmoles sialic acid released by</u>	
	<u>Neuraminidase</u>	<u>Acid hydrolysis</u>
Fetuin (18mg used to couple 1.0 ml sepharose)	4.3	4.9
Fetuin-sepharose (1.0ml)	2.1	3.7

The results indicate that only 60% of the fetuin involved in the coupling reaction is actually attached to sepharose; further, the value of the sialic acid content of fetuin-sepharose as obtained after neuraminidase treatment differs from that obtained after acid hydrolysis. The higher value obtained in acid hydrolysis may possibly be due to agarose hydrolysis products interfering in the Warren assay, while the lower value obtained after neuraminidase treatment may be due to steric hindrance of fetuin-sepharose with the enzyme.

Affinity chromatography of *S. griseus* neuraminidase on fetuin-sepharose

Affinity chromatography was accomplished at 4⁰ C on a column (1.5 x 20 cm) of fetuin-sepharose equilibrated with 0.05 M sodium acetate buffer, pH 5.3. Neuraminidase fraction VI (Fig. 2.5A, Table 2.5) obtained from active fractions after gel filtration on Sephadex G-100 was concentrated by ultrafiltration with a PM-10 membrane and dialysed against 0.05 M sodium acetate ^{buffer}, pH 5.3, before application on to the affinity column. The column was washed with the equilibration buffer until the protein concentration, determined by the method of Lowry et al. [215] was less than 5 μ g/ml (point E, Fig. 2.5B). The bound neuraminidase was then eluted with 0.1 M sodium borate buffer, pH 8.5, containing 0.1 M KCl [206]. The effluent fractions containing neuraminidase were pooled and concentrated by ultrafiltration as above, dialysed against 0.05 M sodium acetate buffer, pH 5.3, and stored as such, or freeze-dried at -15⁰ C until used.

Table 2.5 Purification of neuraminidase from *S. griseus*

Fraction	Procedure	Volume (ml)	Concentration units/ml †	Total activity units	Protein mg/ml	Specific activity u/mg	Yield %	Purification
I	Culture filtrate	17000	0.04	680	0.72	0.056	100	1.0
II	(NH ₄) ₂ SO ₄ (35-65)%*	800	0.33	266.8	4.38	0.075	39	1.34
III	DEAE-cellulose	750	0.27	202.5	2.32	0.12	29.8	2.1
IV	CM-cellulose	110	0.88	96.8	2.0	0.44	14.2	7.9
V	Hydroxylapatite	50	1.56	77.8	0.9	1.73	11.4	30.9
VI	Sephadex G-100	25	2.50	61.3	0.27	9.3	9.0	166.1
VII	Fetuin-sepharose	12	4.40	52.8	0.18	24.4	7.8	435.7

* In these studies the 35-65% ammonium sulphate procedure was found to precipitate more neuraminidase than the 40-60% procedure employed in the original study [43].

† One unit of neuraminidase activity is defined as the amount of enzyme which releases 1 μmole sialic acid per minute in a standard assay of the enzyme.

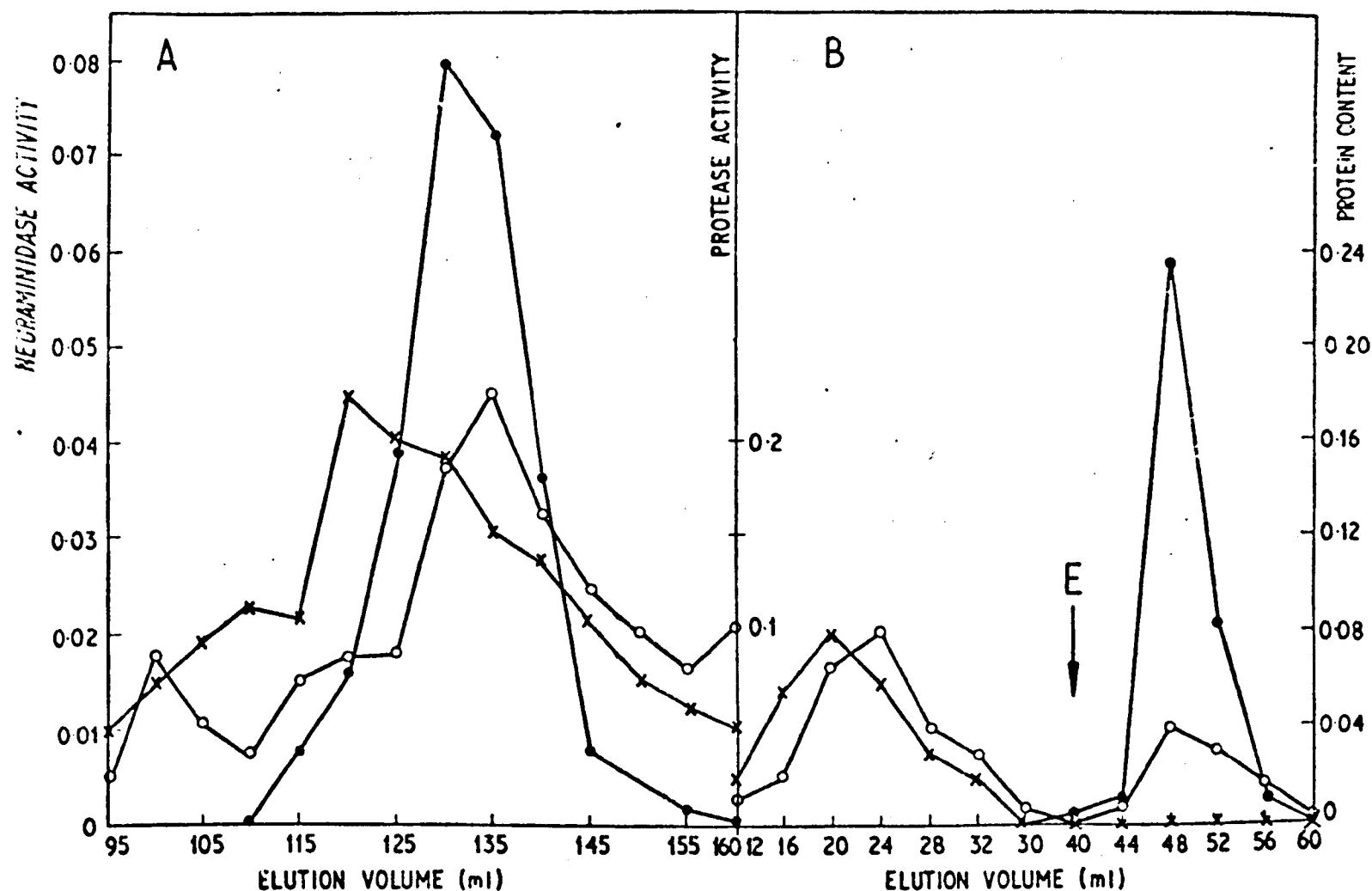


Fig. 2.5 A. Gel filtration on Sephadex G-100 according to Kunimoto *et al.* [43] to produce fraction VI. B. Affinity chromatography on a fetuin-sepharose column as described above. Fraction VI was concentrated to 2.0 ml and applied on the column. Elution was carried out with 0.05 M sodium acetate, pH 5.3, until point E, when 0.1 M sodium borate buffer, pH 8.5, was used to elute the enzyme. (—○—○—) protein concentration (mg/ml)[215]; (—●—●—) neuraminidase activity (μ moles) sialic acid released/0.1 ml); (—x—x—) protease activity (moles bonds cleaved/0.1 ml)[217].

2.2.4 Enzyme purity

Protease assay: The method of Lin *et al.* [217] was used to determine protease activity in the enzyme fractions obtained after gel filtration (fraction VI) and after the final affinity chromatography step (fraction VII). No protease activity was detected in fraction VII (Figure 2.5).

Detection of protease activity by an electrophoretic method

An attempt was made to develop a method in which the intensity of a protein band appearing after its electrophoretic run would reflect on the degree of proteolytic activity to which it was subjected before electrophoresis. Thus, to detect proteolytic contaminants in neuraminidase preparations, the different neuraminidase fractions were each incubated with the same amount of a protein of known purity at 37⁰ C for various times, after which the samples were analysed electrophoretically on 7.5% polyacrylamide gels using the method of Weber and Osborn [218] (Figure 2.6).

From these studies neuraminidase fraction VII was judged free of proteolytic contaminants. While this work was in progress, this idea of detection of proteolytic activity was applied by other workers [220] in peptide mapping studies.

Analytical gel electrophoresis

Further demonstration of purity of neuraminidase was achieved by electrophoresis on 7.5% polyacrylamide-SDS* gels which were prepared and run as described by Weber and Osborn [218] (Figure 2.7) Neuraminidase ran as one single band.

2.2.5 Characterisation of *Streptomyces griseus* neuraminidase

2.2.5.1 Molecular weight determination

(a) The molecular weight of neuraminidase was estimated by gel filtration on a Sephadex G-100 column (2.2 x 50 cm) following the procedure of Andrews [221]. The column was calibrated with five proteins of known molecular weight. Two milligrams of each of these proteins (1.0 mg neuraminidase) was dissolved in 2.0 ml of 0.1% blue

* sodium dodecyl sulphate

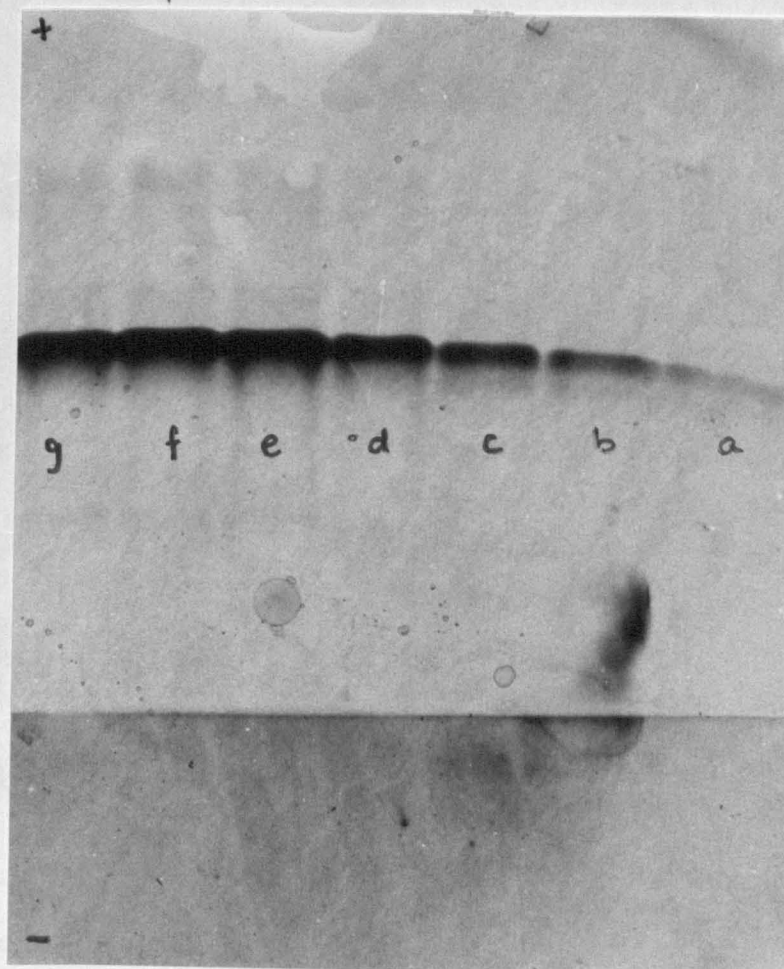


Figure 2.6 Electrophoretic detection of proteolytic contaminants in neuraminidase preparations. The incubation mixtures consisted of 25 μ l of test solution and 60 μ g carbonic anhydrase in 80 μ l of 0.1 M phosphate buffer, pH 7.4 [219]. The bands shown were stained for protein with Coomassie Blue.

- (a) trypsin (20 μ g/ml) incubated for 24 hours
- (b) trypsin incubated for 12 hours
- (c) neuraminidase fraction VI (Table 2.5) incubated for 24 hours
- (d) neuraminidase fraction VI (Table 2.5) incubated for 12 hours
- (e) neuraminidase fraction VII (Table 2.5) incubated for 24 hours
- (f) neuraminidase fraction VII (Table 2.5) incubated for 12 hours
- (g) control containing 25 μ l of buffer instead of test solution incubated for 24 hours

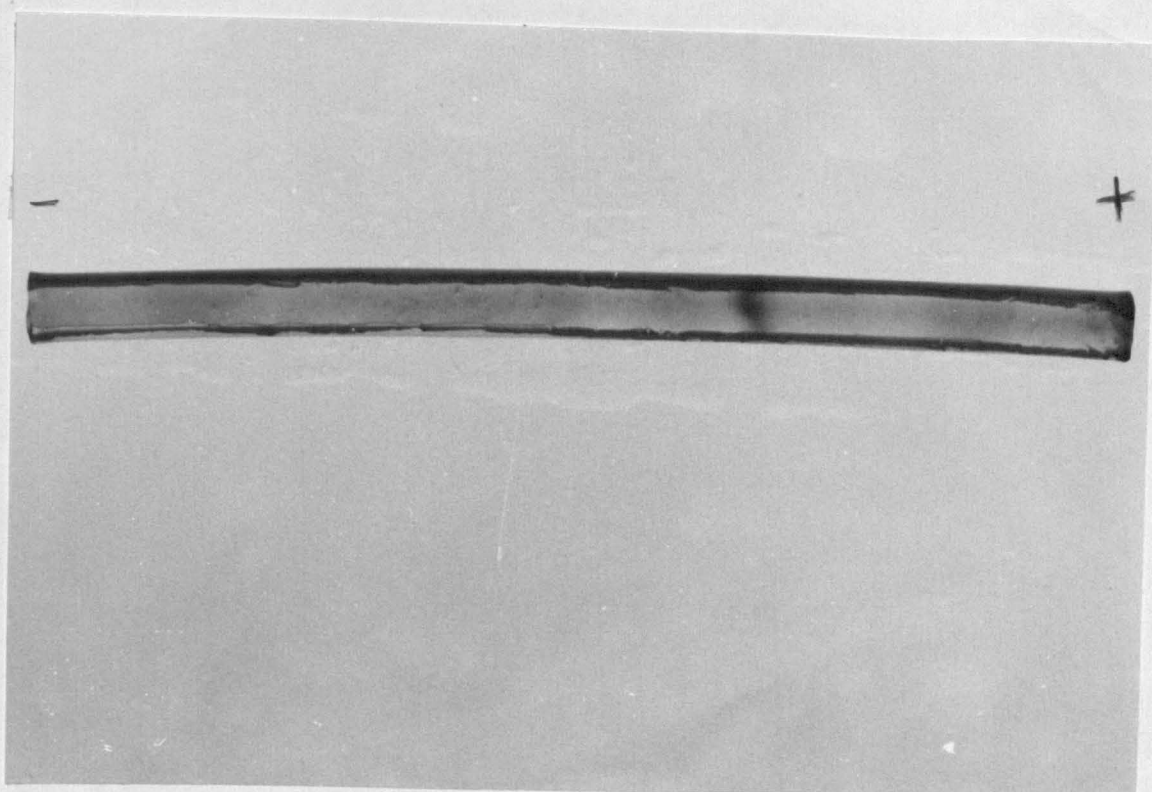


Figure 2.7 Polyacrylamide gel electrophoresis of the neuraminidase purified by affinity chromatography on fetuin-sepharose.

dextran and the solution was slowly eluted from the column with 0.2 M phosphate buffer, pH 7.0, containing 0.05 M KCl at a rate of 40 ml/hour, collecting 4.0 ml fractions. The elution volume of each protein was determined by monitoring the absorbance of the eluted fraction at 280nm. The elution peak of neuraminidase was identified by its enzyme activity (Figure 2.8).

(b) The molecular weight of *S. griseus* neuraminidase was also estimated by SDS-polyacrylamide gel electrophoresis, employing the method of Weber and Osborn [218]. The B.D.H.* molecular weight markers comprising a mixture of oligomers of molecular weights in the range 14,300-71,500 were used as described by the manufacturers [222] along with 15 µg of purified neuraminidase. After the electrophoretic run, the gels were stained with Coomassie blue and destained by diffusion [218] (Figure 2.9).

* British Drug Houses

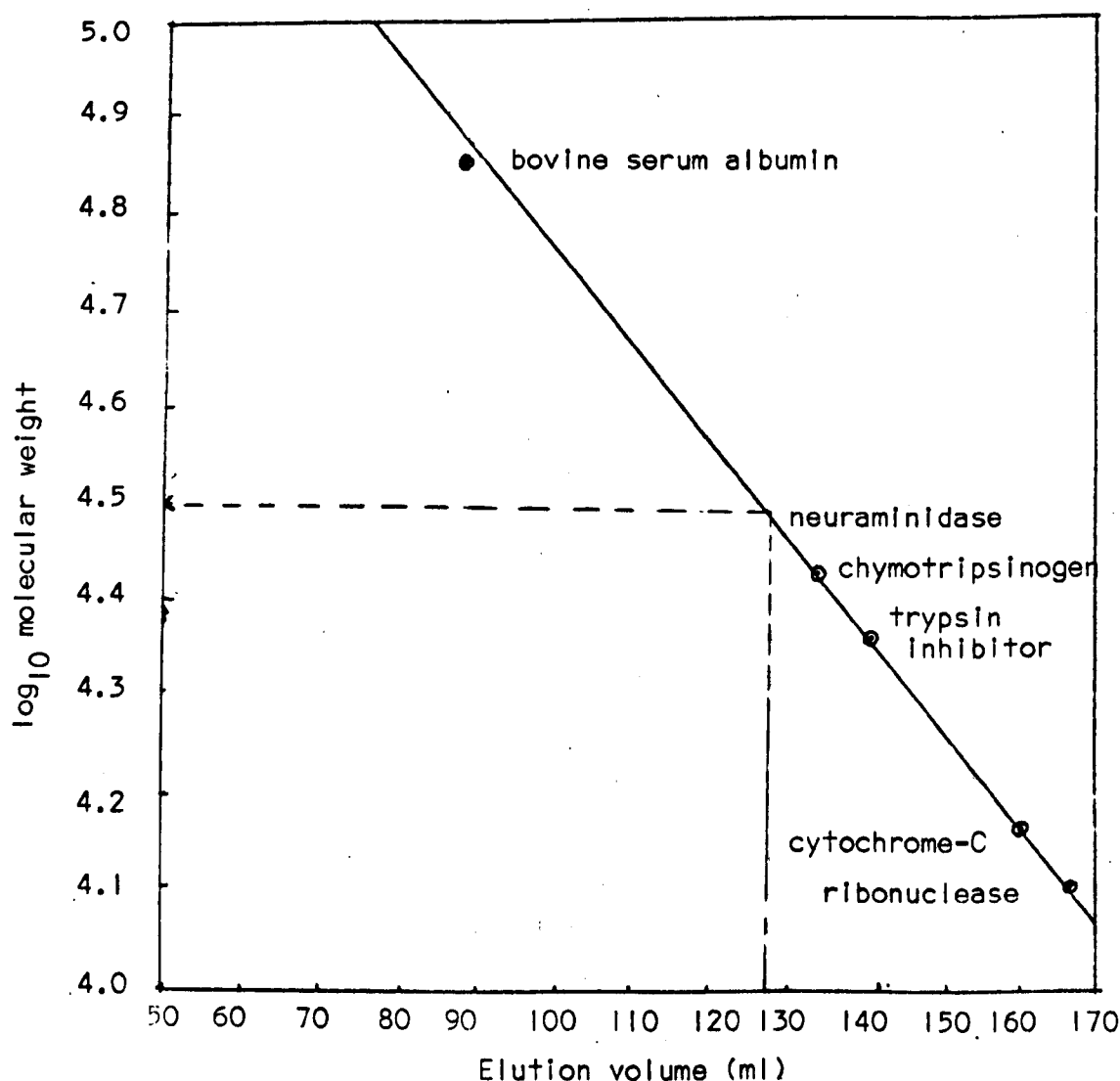


Figure 2.8 Gel filtration of purified *S. griseus* neuraminidase on a Sephadex G-100 column (2.2 x 50 cm).

The molecular weight determined from gel filtration was 32,000 while that obtained from electrophoresis was 31,600. A value of 34,000 was kindly estimated by Dr. I. Kennedy after ultracentrifuge sedimentation of the purified neuraminidase on a sucrose gradient.

Table 2.6 Molecular weight determination of neuraminidase by electrophoresis

Band	M. Wt	$\log_{10} \frac{M. Wt}{M. Wt}$	Distance band from start (cm)	Electrophoretic mobility
Ref. Bromophenol blue	-	-	14.6	100
a	14,300	4.15	13.1	89.7
b	28,600	4.45	9.5	65.1
c	42,900	4.63	6.9	47.3
d	57,200	4.76	5.1	34.9
e	71,500	4.85	4.0	27.4
f	85,800	4.93	3.0	20.5
N	31,623	4.5	9.0	59.5

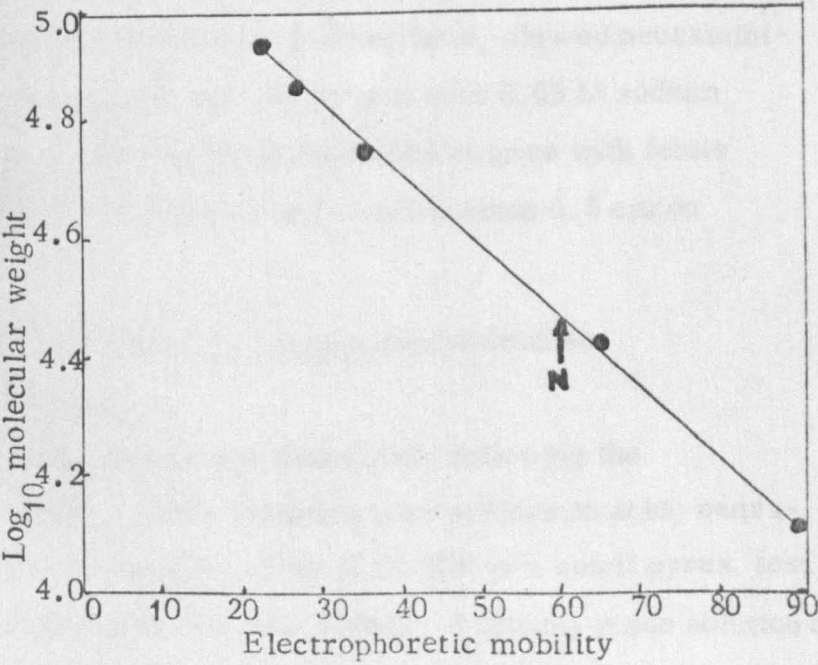
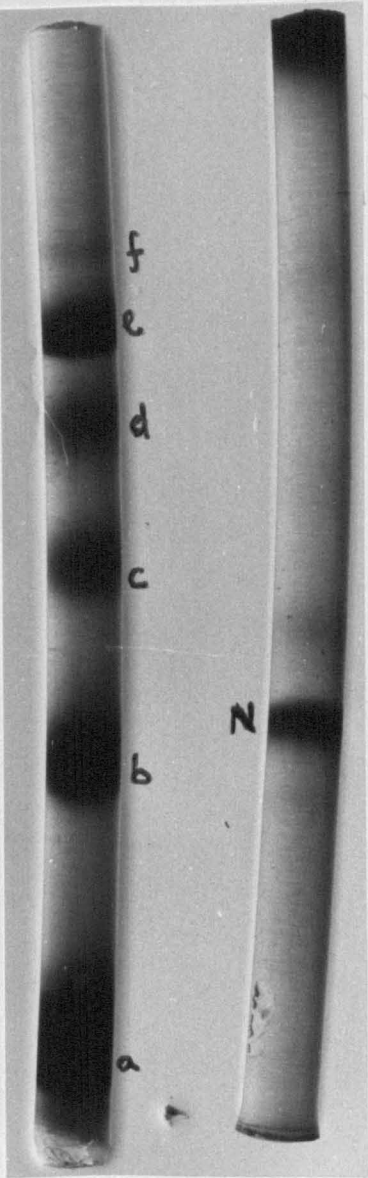


Figure 2.9 Determination of the molecular weight of *S. griseus* neuraminidase by SDS-polyacrylamide gel electrophoresis. a-f correspond to the bands whose molecular weights are shown in the table (Table 2.6).
N - neuraminidase.

(c) Electrophoresis of neuraminidase in non-denaturing conditions.

Electrophoresis of purified *S. griseus* neuraminidase was carried out employing the method described by Gabriel [223]. Neuraminidase (previously dialysed against 0.05 M Tris-phosphate buffer, pH 7) was applied as a sample (containing 200 µg in 100 µl of dialysis buffer and 0.0002% methylene blue) and run on a cylindrical 7.5% polyacrylamide gel (0.5 x 10 cm) using a current of 1.5 mA per tube. The electrophoresis was conducted at 4°C, after which the gels were longitudinally sliced and stained for protein [218] or neuraminidase activity [224]. The staining for neuraminidase activity using 2, (3'-methoxyphenyl)- α_1 -N-acetylneuraminic acid (MPN) and Black K salt (a diazonium salt of 4-amino-2,5-dimethoxy-4-nitroazobenzene) was not successful, but one of the 0.50 cm transverse sections of the gel, corresponding to the protein stained band, showed neuraminidase activity after eluting the enzyme from it with 0.05 M sodium acetate buffer, pH 5.3, and assaying the eluted enzyme with fetuin as described earlier. Neuraminidase travelled about 3.5 cm on the separation gel.

2.2.5.2 Chemical analysis of *S. griseus* neuraminidase

(a) N-terminal analysis

The N-terminal amino acid was determined following the procedure of Gray [225]. After oxidation with performic acid, neuraminidase (0.3 mg) was dissolved in 50 µl 1% SDS in a small pyrex test tube and 50 µl N-ethylmorpholine was added. A freshly made solution of dansylchloride (25 mg/ml, 75 µl) in anhydrous dimethyl formamide was added and mixed; the reaction was allowed to proceed at room temperature for 3 hours after which the labelled enzyme was precipitated by adding acetone (0.6 ml) to the reaction mixture. The resulting precipitate was compressed by centrifugation, washed with 80% acetone (500 µl), centrifuged again, dried by evaporation in vacuo and hydrolysed with 6 N HCl (150 µl) at 110°C for 18 hours. The resulting dansyl amino acids were identified chromato-

graphically by comparison with dansyl amino acid markers on polyamide layer sheets employing the solvent systems of Hartley [226]. The dansylated amino acids were visualised under ultra-violet light and the position of the spot corresponding to the dansyl alanine marker coincided with that of a spot arising from the hydrolytic products of the dansylated neuraminidase. Alanine was therefore judged to be the N-terminal amino acid in this enzyme.

(b) Amino acid and sugar analysis*

Amino acid analyses of the purified neuraminidase from Streptomyces griseus were done on a Locarte automatic acid analyser; neutral sugars were analysed on a Jeol JLC-6AH sugar analyser. The values for the amino acid content of neuraminidase are given in Table 2.7 and are the mean of four determinations, based on the molecular weight of 32,000.

Table 2.7 Amino acid content of S. griseus neuraminidase

Amino acid	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Cys	
No. residues	23.3	17.3	17.9	38.6	38.6	39.7	21.1	1.3	
Amino acid	Val	Met	Ileu	Leu	Tyr	Phe	His	Lys	Arg
No. residues	18.9	5.4	12.4	19.3	7.0	8.3	9.0	17.3	9.9

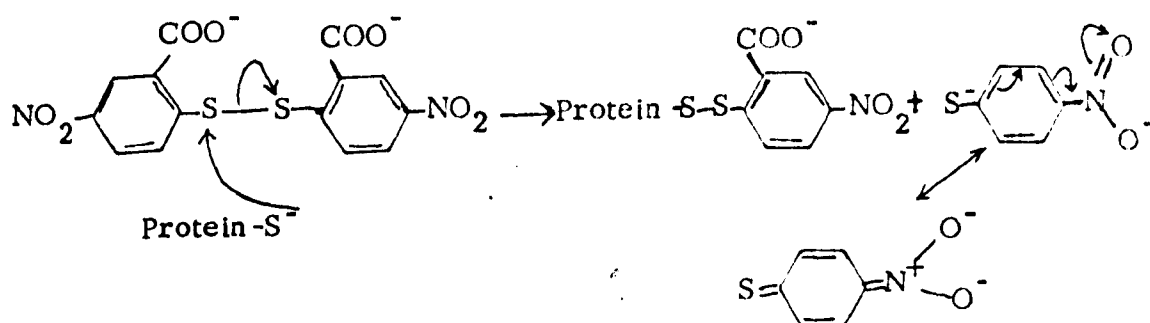
The value for tryptophan was not determined; however, Augustus [227], working on the same enzyme, has reported a maximum of 2 residues. In the neutral sugar analysis, mannose, galactose, xylose, fucose, fructose and arabinose were detected in trace amounts. In contrast, the glucose content was about 20 residues per molecule of neuraminidase. Since only two determinations were made, these values are not

conclusive. The high value of glucose may have been derived from the chromatographic supports employed in the course of enzyme purification. Further acidic and basic sugars were not determined.

* Amino acid and sugar content was determined by Dr. J.E. Fox, Macromolecular Analysis Service, Birmingham University.

(c) Determination of disulphide links in *S. griseus* neuraminidase

The method of Cavallini et al. [228] using sodium borohydride in 8 molar urea as the reducing agent and 5, 5'-dithiobis(2-nitrobenzoic) acid (DTNB) as a thiol disulphide exchanger was used to estimate the total number of sulphydryl groups in neuraminidase. Fig. 2.10 shows the titration of sulphydryl groups in different amounts of the enzyme.



In the reaction at pH 8, anionic forms of protein thiol groups attack the disulphide bond of the DTNB reagent expelling a stable-coloured nitrothiophenolate anion which may be estimated spectrophotometrically at 412 nm.

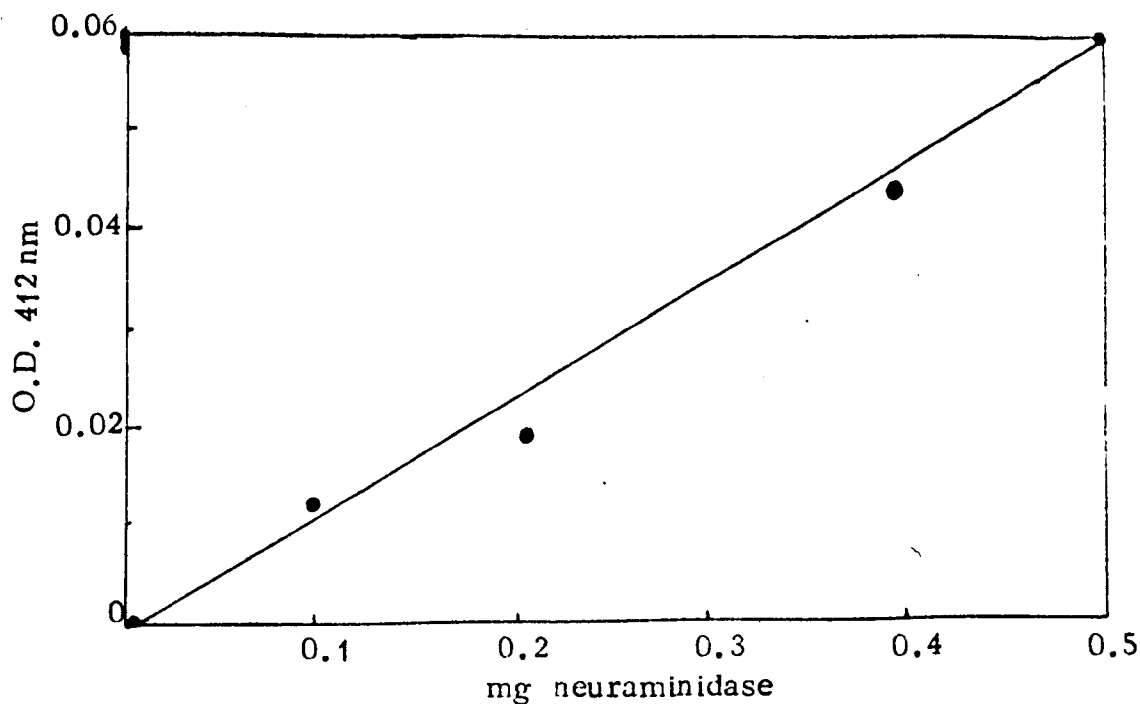


Fig. 2.10 Determination of disulphide groups in neuraminidase with DTNB.

The number of total sulphydryl groups after reduction is given by the formula

$$N = \frac{M.wt. \times A \times V}{12,000 \times m}$$

where M.wt. is the molecular weight of the enzyme (32,000 in this case); A is the optical density value obtained at 412 nm in the method; V is the volume of the final solution (6.0 ml) and m is the weight (mg) of the enzyme sample analysed. The figure 12,000 is the molar extinction coefficient of the nitrothiophenolate anion.

$$\therefore N = \frac{32,000 \times 6 \times A}{12,000 \times m}$$

($\frac{A}{m}$) is the slope of the graph (Fig. 2.11) relating values of A and the weight of neuraminidase. In this case ($\frac{A}{m}$) = $\frac{0.030}{0.275}$,

$$\therefore N = \frac{0.030}{0.275} \times \frac{32,000 \times 6}{12,000} = 1.82$$

This number of sulphydryl groups, however, includes the pre-existing sulphydryl groups.

(d) Determination of sulphydryl groups in *S. griseus* neuraminidase

The technique for the quantitative determination of sulphydryl groups in proteins developed by Rohrbach et al. [229] using 4,4'-bis - dimethylaminophenyl carbinol (BDC-OH)* was applied on neuraminidase. Neuraminidase (1.6 mg) was dissolved in 1.0 ml 0.04 M sodium acetate buffer, pH 5.1, containing 4 M guanidine hydrochloride. Aliquots of 50, 100, 150, 200 and 250 μ l of this solution were separately mixed with 100 μ l of 0.65 mg/ml solution of BDC-OH in acetone and the mixture was made up to 5.0 ml with acetate buffer. After 30 mins the absorbance of each solution was determined at 612 nm (Fig. 2.11).

* 4,4'-bis - dimethylaminophenyl carbinol (BDC-OH) was synthesised by L. Carr following the method reported by Rohrbach et al. [229].

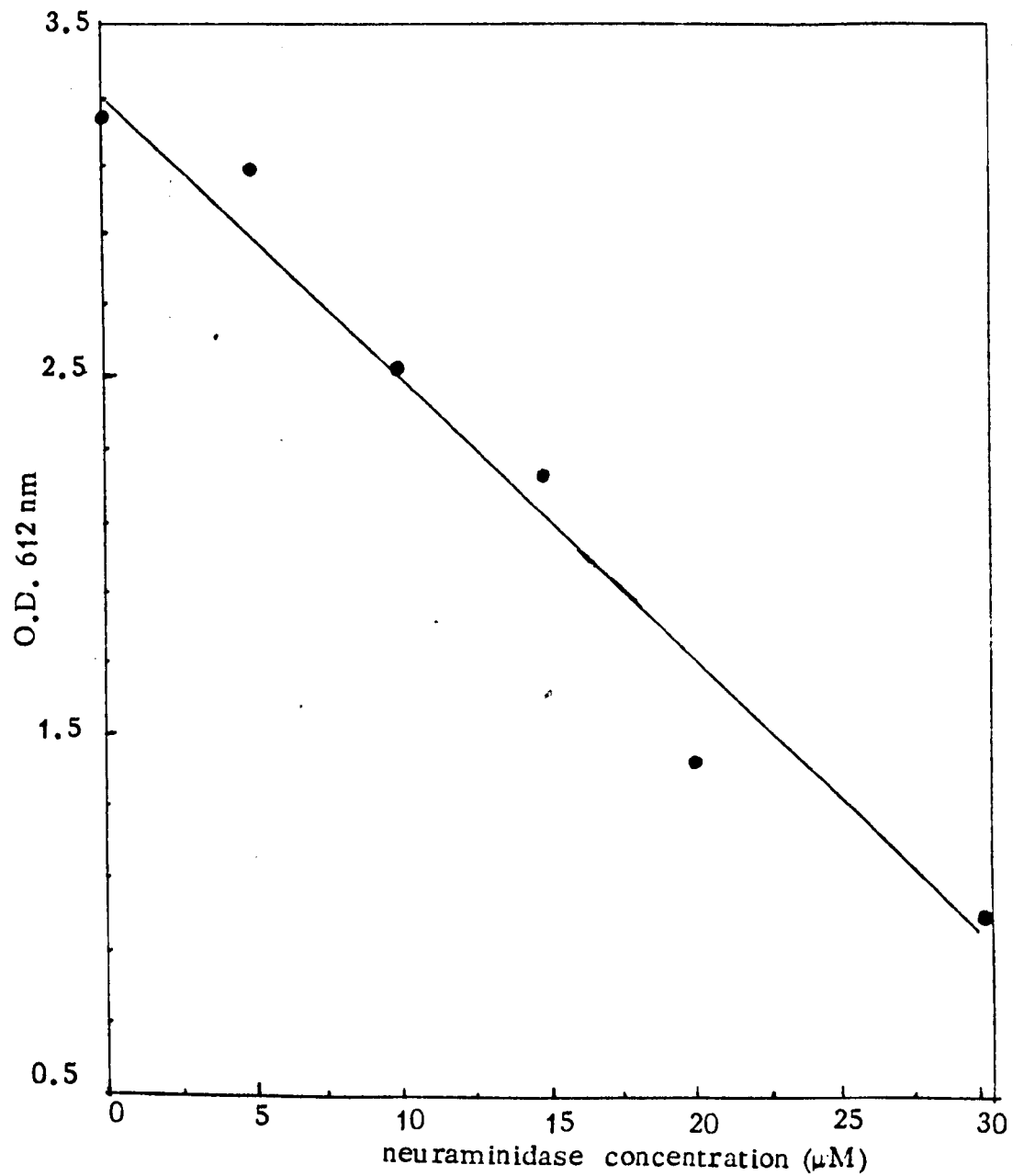


Fig. 2.11 Determination of sulphydryl groups in neuraminidase

Number of sulphydryl groups = $\frac{-\text{slope}}{E_m}$ (where E_m is the molar extinction coefficient of the reacted BDC-OH = $70,800 \text{ M}^{-1} \text{ cm}^{-1}$),

$$\therefore \text{the number of sulphydryl groups} = \frac{-1.6}{70,800 \times 17.5 \times 10^{-6}}$$

$$= 1.29$$

(e) Demonstration of glycoprotein nature of *S. griseus* neuraminidase

Two methods of staining glycoproteins were used to detect carbohydrate in protein bands corresponding to neuraminidase after gel electrophoresis. The periodic-acid-Schiff (PAS) procedure of Kapitany and Zebrowski [230] and the sensitive fluorescent staining method of Eckhardt et al. [231] were used. A device of obtaining longitudinal slices of cylindrical polyacrylamide gels was designed to permit easier comparison of the protein- and carbohydrate-stained bands on gels. This device is described in Appendix 3; here it will suffice to mention that the device obviates the necessity to run two samples (to be stained differently) or to employ other methods which lack precision, e.g. slicing with a scalpel or wire.

After electrophoresis as described earlier, the gel on which purified neuraminidase was run was sliced longitudinally and one half of the gel was stained for protein (Fig. 2.7) while the other half was stained for glycoprotein. Figure 2.12 shows the fluorescent band of neuraminidase demonstrating the glycoprotein nature of this enzyme.

2.2.6 Catalytic properties of *S. griseus* neuraminidase

The effect of enzyme concentration, time, temperature and pH on the activity of the purified *S. griseus* neuraminidase was studied, using sialyllactose, α_1 -acid glycoprotein, fetuin and mucin as substrates. The effect of various metal ions and the inhibitory effect of N-acetyl neuraminic acid on the enzyme activity were also investigated.

The assay mixtures consisted of the substrate solution (0.4 ml) in 0.05 M sodium acetate buffer, pH 5.3, (1 mg/ml sialyllactose; 4 mg/ml α_1 -acid glycoprotein; 5 mg/ml fetuin; 2 mg/ml mucin), 0.05 M sodium acetate buffer (0.4 ml) and neuraminidase solution (0.2 ml). In blank incubations the enzyme solution was replaced by an equal volume of buffer while in studies on the inhibitory effect of N-acetylneuraminic acid, MPN* (0.5 mg/ml) was used as the substrate. After appropriate

* 2-(3'-methoxyphenyl)-d₁-N-acetylneuraminic acid

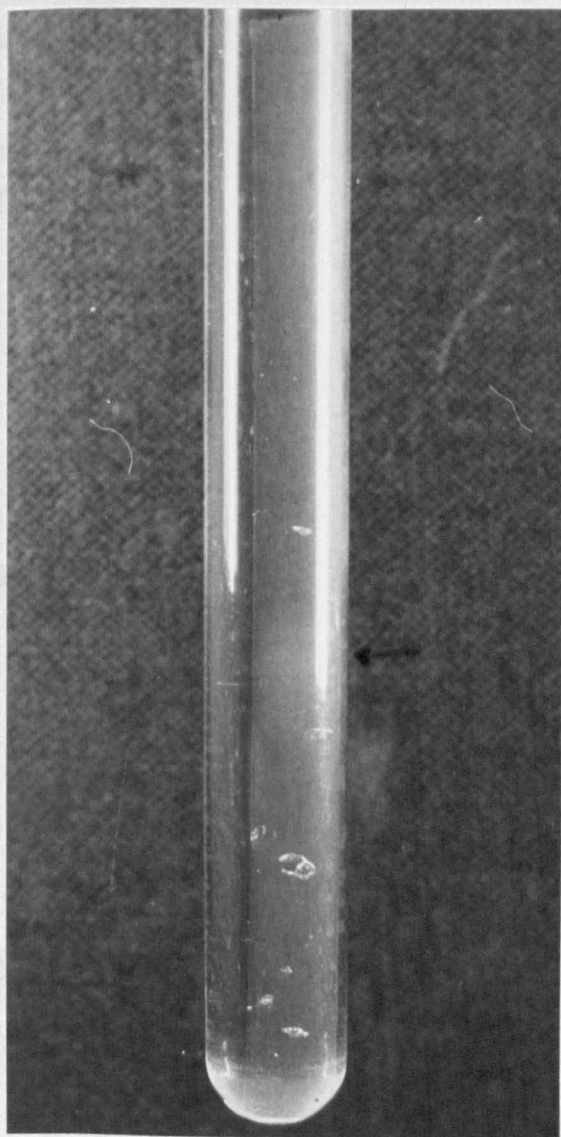


Fig. 2.12

Neuraminidase electrophoretic band stained with dansyl hydrazine [231]. The arrow shows the fluorescent band corresponding to the protein band of neuraminidase.

times of incubation, the released sialic from each substrate was determined by the thiobarbituric acid procedure [196] except in the case of MPN when the enzymic activity was followed by determining the released 3-methoxyphenol as described by Palese et al. [224] using the Folin phenol test. The results obtained in these studies are summarised in Figures 2.13-2.15 and Tables 2.8 and 2.9.

The kinetic constants (K_m and V_{max}) of *S. griseus* neuraminidase were determined using various amounts of sialyllactose; the assay of the enzyme was conducted as described above. The initial rate of hydrolysis at each substrate concentration was determined and the results obtained were analysed by the method of Lineweaver and Burk [232] (Figure 2.17).

The protein concentration of neuraminidase used in the assay mixtures was 0.5 mg/ml. Hydrolysis of various substrates was carried out for different periods, after which the amount of sialic acid released from each substrate by incubation with neuraminidase at 37° C was determined by thiobarbituric acid procedure [196].

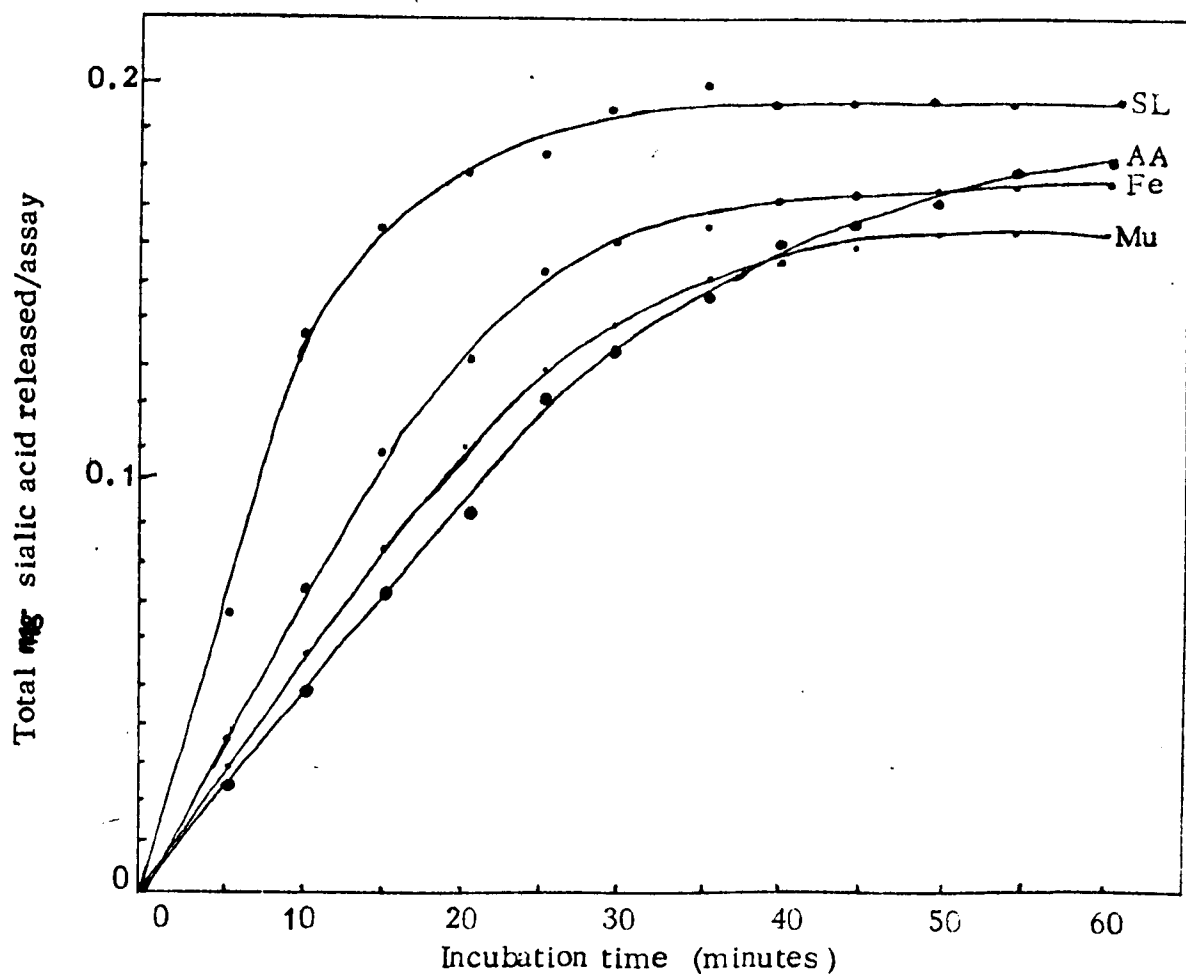


Figure 2.13 Hydrolysis of various substrates by *S. griseus* neuraminidase. SL, sialyllactose; AA, α_1 -acid glycoprotein; Mu, mucin; Fe, fetuin. Reaction conditions as in text.

To determine the effect of metal ions on the enzyme, the assay system already described was used but with substrates previously exhaustively dialysed against 0.005 M sodium acetate buffer, pH 5.3, at 4° C. The activity of the enzyme in the reaction mixtures supplemented with metal ions (Table 2.8) was compared with the unsupplemented control; it was found that the substances added had no substantial effect on the initial rate of neuraminidase activity except Hg²⁺ and Fe²⁺ which slightly inhibited the enzyme. These observations agree with those of Kumimoto et al. (43) for the same enzyme, but differ from the *vibrio cholerae* and the viral enzyme (33, 34, 187) which are dependent on metal ions for activity.

Table 2.8 Effect of added metal ions on neuraminidase activity

Substance added	Concentration Mx 10 ⁻³	%control activity
Control	-	100
CaCl ₂	0.5	90
MnCl ₂	0.5	100
CoCl ₂	1.0	97
MgCl ₂	1.0	89
FeCl ₂	0.5	65
ZnCl ₂	0.8	90
HgCl ₂	0.5	55
Fe ₂ Cl ₃	0.5	91

When investigating the effect of temperature on *S. griseus* neuraminidase the enzyme solution (0.5 mg/ml) to be assayed was exposed to the temperatures shown (Table 2.9) for various times (A, 5 minutes ; B, 10 minutes; C, 30 minutes; D, 2 hours) and then assayed as described, using fetuin as the substrate and an incubation period of 50 minutes.

Table 2.9 Effect of temperature on *S. griseus* neuraminidase

Temperature ° C	0	37	45	50	60
% full enzymatic activity					
A	100	100	65	10	3
B	100	95	55	3.5	0
C	100	86	32	1.4	0
D	100	60	15	0	0

To determine the effect of pH on the enzyme, the assay mixtures containing the substrate solution (0.5 ml), the buffer (0.3 ml, 0.1 M sodium acetate or 0.1 M sodium phosphate) and enzyme solution (0.2 mg/ml, 0.2 ml) were incubated at 37° C for various times, after which the amount of sialic acid released in each case was determined, and the corresponding enzyme rates were compared as a function of pH (Figure 2.14).

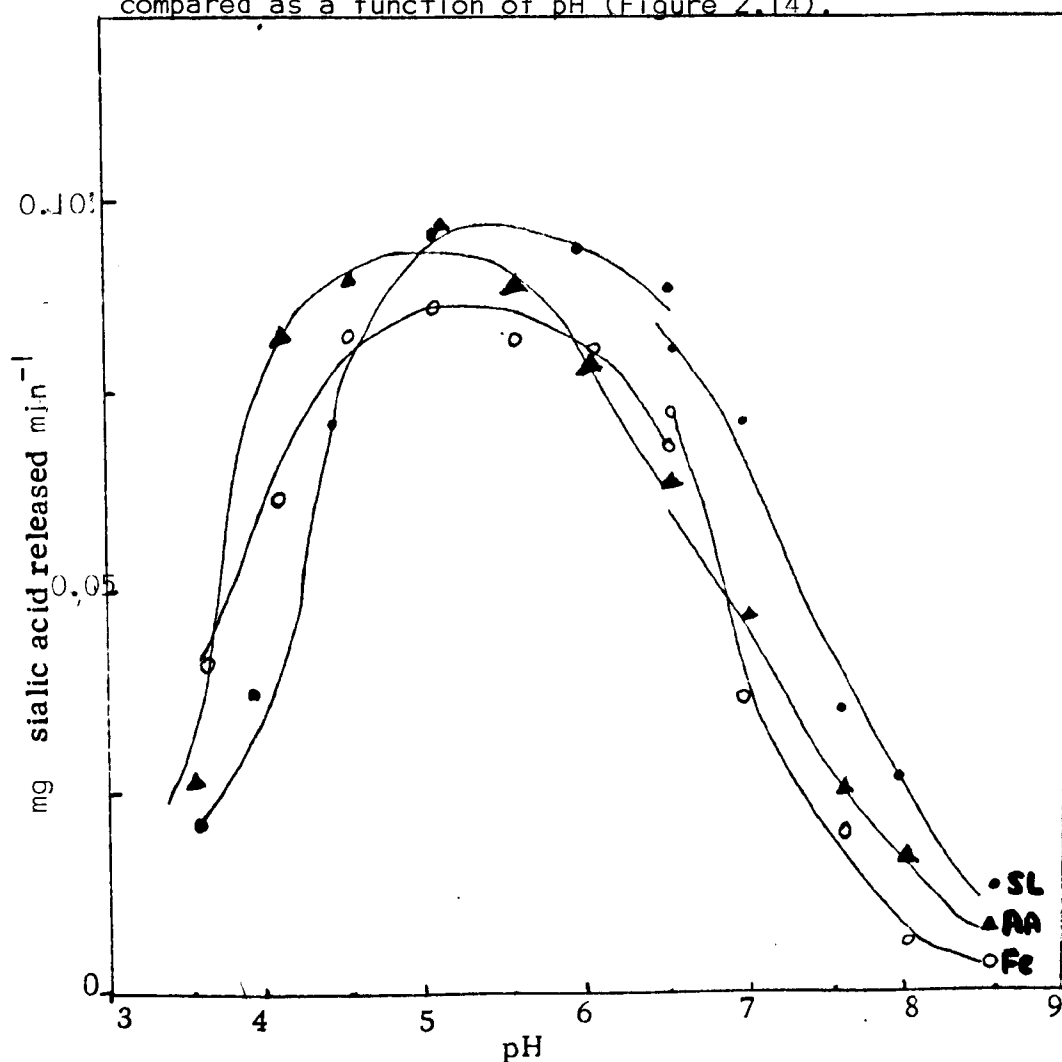


Figure 2.14 Effect of pH on reaction rate of hydrolysis of sialyl-lactose (SL), α_1 -acid glycoprotein (AA), fetuin (Fe) by *S. griseus* neuraminidase. pH 3-6.5 using acetate buffer; pH 6.5-8 using phosphate buffer.

When studying the inhibition of *S. griseus* neuraminidase by N-acetyl neuraminic acid (NANA), the enzyme was assayed using 2-(3'-methoxyphenyl)-N-acetyl- α -neuraminic acid (MPN) and determining the released 3-methoxyphenol [224]. The enzyme solution (0.5 mg/ml, 0.3 ml) was incubated at 37°C with MPN (2×10^{-3} M, 0.5 ml) and the rate of release of 3-methoxyphenol in the presence of varying amounts of NANA in a total volume of 1.0 ml were determined. The rate of 3-methoxyphenol release in the control (in the absence of NANA) was taken as 100% of enzyme activity and the activities of the enzyme thus determined in the presence of NANA were expressed as % control enzyme activity (Fig. 2.15).

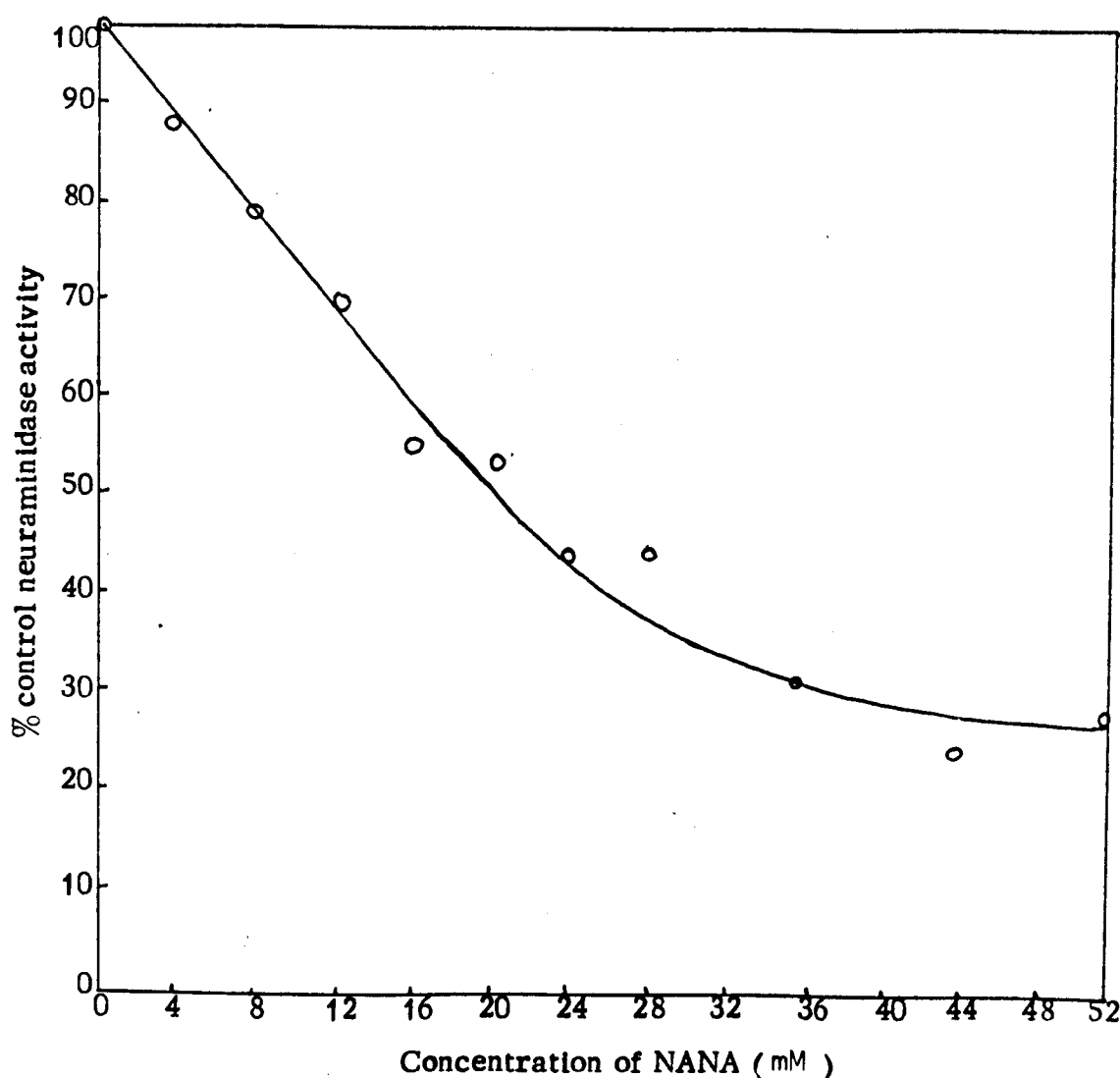


Fig. 2.15 Inhibition of *S. griseus* neuraminidase by N-acetyl-neuraminic acid (NANA). The shown amounts of NANA were incubated with the enzyme for 5 minutes before the substrate (MPN) was added and the enzyme activity determined.

To determine the nature of the inhibition of *S. griseus* neuraminidase by NANA, neuraminidase (0.5 mg/ml, 0.5 ml) and MPN (2×10^{-3} M, 0.5 or 1.0 ml) were incubated at 37° C with varying amounts of NANA in a total volume of 2.0 ml. At various time intervals (starting at zero time) up to 200 μ l were withdrawn and the amount of 3-methoxyphenol produced was immediately determined. A rate curve was thus obtained for each NANA concentration and for both levels of substrate concentration. The results obtained were plotted according to Dixon (240) (Figure 2.16).

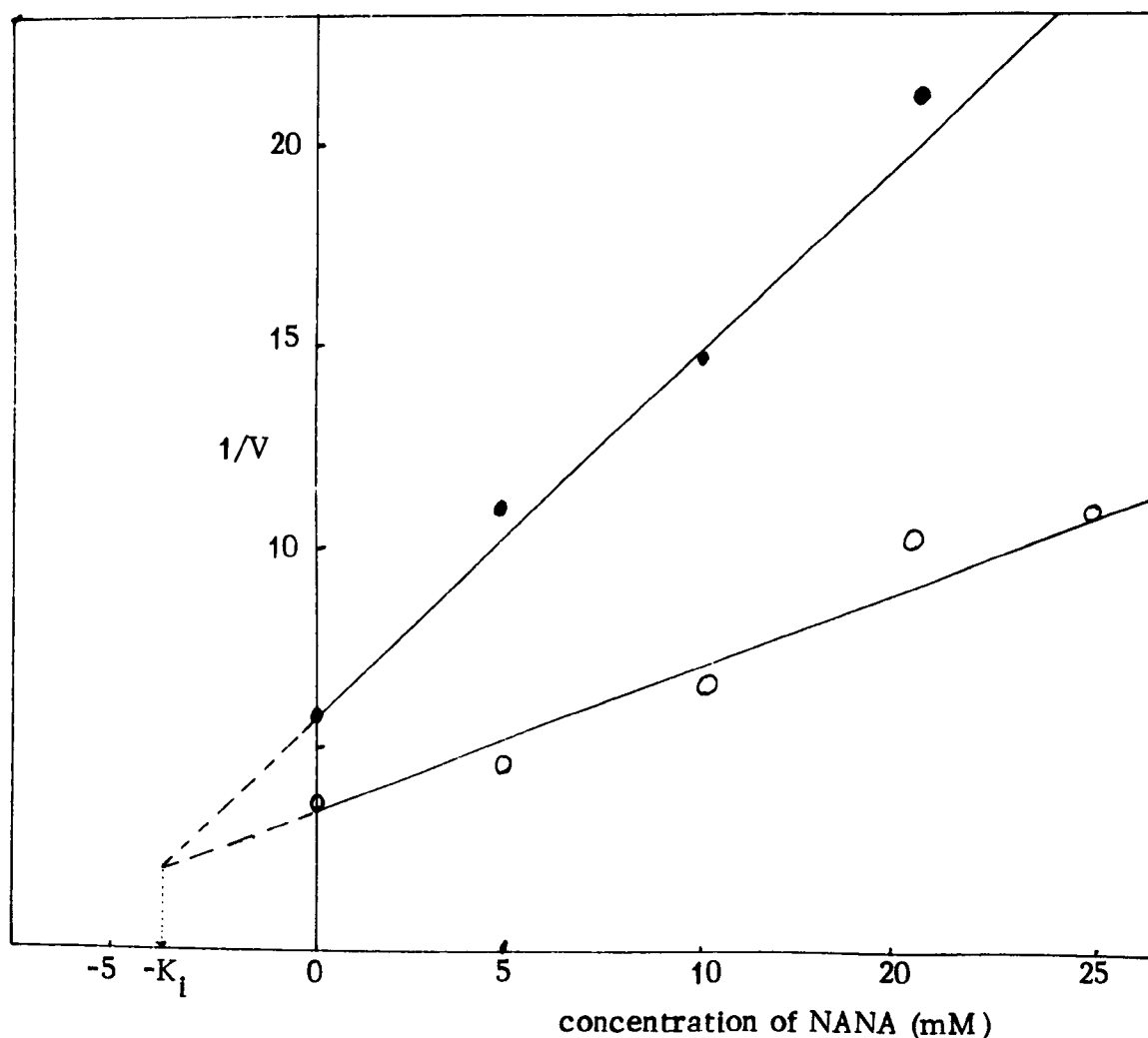


Fig. 2.16 Dixon plot for the determination of the type of *S. griseus* neuraminidase inhibition by NANA. The values on the $1/V$ axis are reciprocals of the amounts of 3-methoxyphenol released per unit time concentration. NANA, / (—●—●—) 0.5 ml, (—○—○—) 1.0 ml of MPN solution. A value of -4.0 mM was estimated for K_i , the inhibitory constant.

S. griseus neuraminidase (0.5 mg/ml, 0.2 ml) was incubated with varying amounts of sialyllactose in a total volume of 1.0 ml

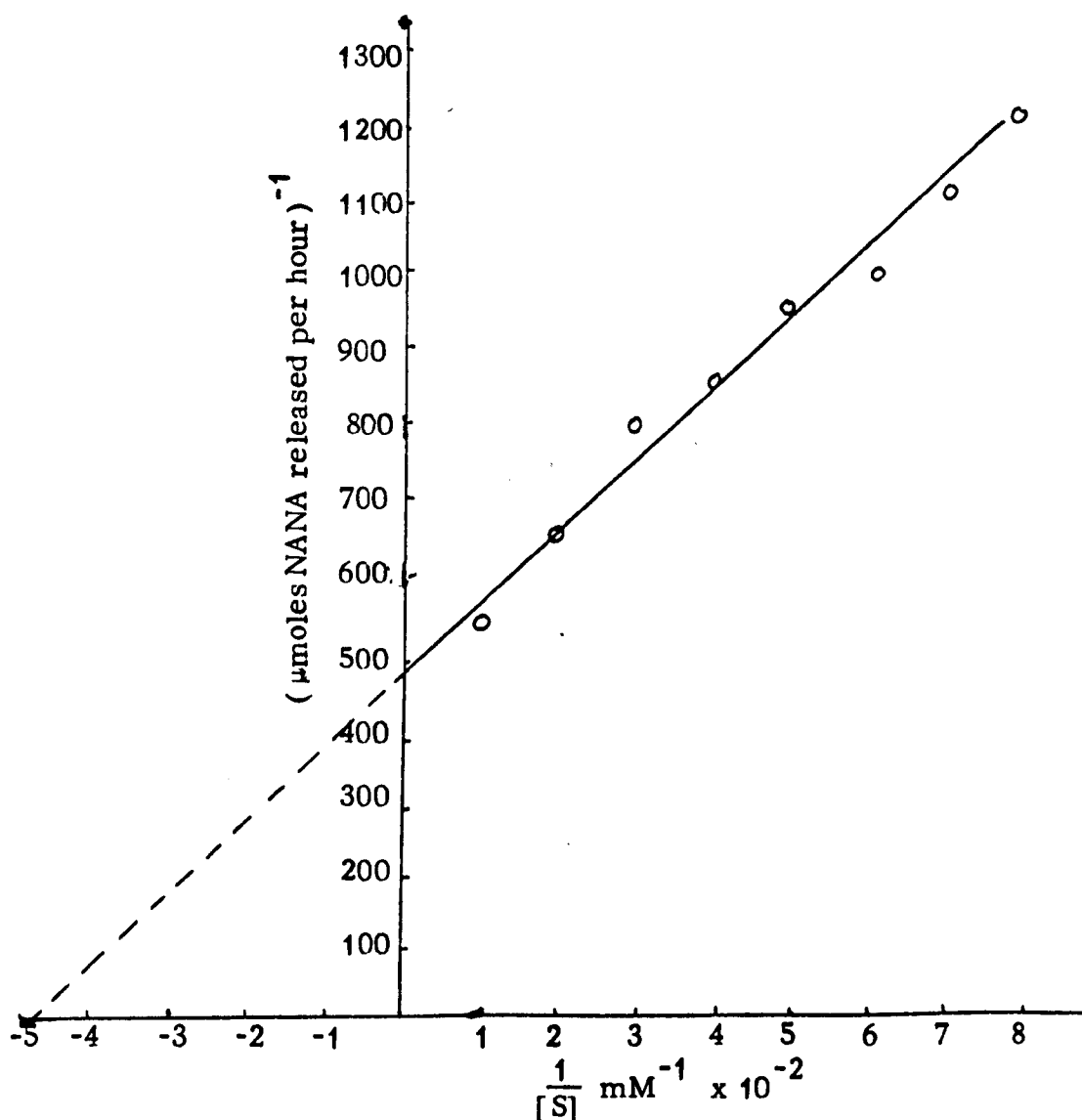


Figure 2.17 Determination of K_m and V_{max} of S. griseus neuraminidase; using sialyllactose, these values were respectively $1.8 \times 10^{-4} \text{ M}$ and $0.021 \mu\text{moles}$ N-acetylneuraminic acid per μg enzyme protein.

and for each concentration of sialyllactose used, the initial rate of neuraminidase reaction was determined and the results thus obtained were analysed according to the method of Lineweaver and Burk (232) (Figure 2.17).

2.2.7 Discussion

As mentioned earlier, the more potent neuraminidase producers, e.g. Clostridium perfringens, Vibrio cholerae are known pathogens; the pathogenicity of Streptomyces griseus has not been reported in man. However, Streptomyces have been known to cause pneumonia in buffaloes [233] and some strains have been shown to produce a volatile substance which kills fungi [234]. It is also noteworthy that some species of Streptomyces produce a specific inhibitor of viral neuraminidases (called neuraminin) which is without effect on bacterial neuraminidases [235].

The production and induction of neuraminidase in several species of Streptomyces has been reported [41, 43, 213]. In the present studies neuraminidase was produced from S. griseus by induction. Over 4 times more neuraminidase was produced with than without added inducer. Neuraminidase appeared in the culture fluid in amounts corresponding to the increase in cell mass; the enzyme activity started decreasing when the maximum point of growth (after about 72 hours) was reached. There was a slight increase in the extent of growth (related to cell number) in the induced over the non-induced cultures (Figure 2.4), but it is difficult to judge whether the greater production of neuraminidase in induced cultures was a result of an increase in cell number or due to actual induction of the enzyme.

More neuraminidase was produced when α_1 -acid glycoprotein (rather than fetuin) was used as an inducer. This may be attributed to the higher sialic acid content of the former and also possibly to the presence of multiple glycosidic linkages, some of which are resistant to neuraminidase, and thus favour the persistence of α_1 -acid glycoprotein in solution as an inducer. It was interesting to note that the fetuin-induced neuraminidase released sialic acid from fetuin much faster than the α_1 -acid glycoprotein induced enzyme or the enzyme produced from non-induced cultures. Although these

observations were not repeated in induction studies other than the two reported above, the possibility of inducing neuraminidase specifically is interesting. It would be possible to study the apparent lack of specificity exhibited by neuraminidases in cleaving (even if at different rates) most possible glycosidic linkages of sialic acid to its natural partners. However, these results are in contrast to the work of Uchida *et al.* [41] in which the neuraminidases induced by colominic acid (a homopolymer of sialic acid with α , 2-8 linkages) in several bacteria including *Streptomyces* were shown to possess wider substrate specificities.

The gel filtration method introduced to remove low molecular weight substances which interfere in the thiobarbituric acid test for the released sialic acid also served to remove substances such as carbohydrates, which react with the Lowry reagent in the same manner as proteins [236]. The use of both substrate and enzyme blanks (S_0 and E_0 respectively) in assaying neuraminidase in culture fluids further served to exclude interference from the contaminants remaining after the gel filtration step. Nevertheless, the values for neuraminidase content obtained were often not reproducible; as well as inconsistencies in the assay procedure, the cultures of *S. griseus* may have somehow varied in their ability to produce the enzyme.

Various other methods have been used to exclude interfering substances in the assay for neuraminidase, especially in crude biological materials. Srivastava and Abou-Issa [237] added ammonium pyrrolidine dithiocarbamate before the periodate oxidation step in order to eliminate chromogen formation due to reactive uncleaved sialic acid from glycoproteins, but the mechanism by which this is done is not known. Recently Santer [238] described a new method of measuring neuraminidase activity by reacting the enzymatically released aglycone from 2-(3-methoxyphenyl)- α -D-neuraminic acid with amino-antipyrine in the presence of potassium ferricyanide to yield a

coloured complex ($E_m = 11,000$). Although this method shows great promise of use on account of its simplicity and rapidity, it is not more sensitive than the Warren assay ($E_m = 57,000$), besides, since the colour development depends on the effect of an oxidising agent, it would be subject to interference from indigenous reducing or oxidising agents in biological samples. The other methods of assaying neuraminidase (mentioned at the beginning of this chapter) are also limited in use because, although they are more sensitive than the Warren assay, they require additional procedures such as column chromatography or dialysis, or depend on substrates which are not available commercially or demand elaborate and expensive equipment.

The neuraminidase produced by *S. griseus* was purified to homogeneity. The enzyme ran as a single band on SDS-polyacrylamide gel electrophoresis and did not contain any contaminating proteases. Treatment of the polyacrylamide gel, after electrophoresis of neuraminidase, with periodate/dansyl hydrazine or periodate/Schiff's reagent detected a carbohydrate band coincident with the protein-stained band of neuraminidase, and together with the results of the sugar analysis indicated that this enzyme is a glycoprotein.

A molecular weight of 31,600 was assigned to the enzyme by electrophoretic analysis while 32,000 was the value estimated by gel filtration and 34,000 was determined by ultracentrifugation. All these values of molecular weights are close to the range of 31,000-33,000 determined by Kunimoto et al. [43] by gel filtration; further, their concordance would suggest that the enzyme is a single polypeptide. However, because of the glycoprotein nature of the enzyme, these values may be anomalously higher than their real values as would be expected for polypeptides of the same size [221, 239].

From the amino acid analysis, the rather high content of serine and threonine (together constituting 12.6% of the amino acid residues) may be relevant to the glycoprotein nature of the enzyme. Other neuraminidases [210, 212] also possess high amounts of serine and threonine and have been shown to be glycoproteins.

The acidic residues (22%) outnumber the basic ones (12%). This is also true with other neuraminidases whose amino acid content is known [187, 210, 212]. However, in the case of S. griseus neuraminidase this property neither is consistent with the enzyme's failure to bind to a DEAE-cellulose column at pH 6 while it binds fairly tightly to a CM-cellulose column at pH 5.3, nor does it explain the high isoelectric point of 7.9-8 determined by Kunitomo et al. [43]. However, in the study (reported above) in which the electrophoresis of this neuraminidase was run in the absence of SDS in non-dissociating conditions at pH 7.0, the enzyme behaved as a negatively charged protein and migrated to the anode. This would suggest that the isoelectric point of the enzyme is at least below pH 7.0 since proteins are negatively charged at pH's above their isoelectric point. Again, the anomalous behaviour of glycoproteins in these studies or the nature and content of the basic and acidic sugars (which were not determined) may be responsible for the inconsistencies observed. The high dicarboxylic acid content would justify the use of a hydroxylapatite column in the purification of the enzyme. The enzyme has very low content of sulphur-containing amino acids, and the observed lack of effect by p-chloromercuribenzoate [43] on the enzyme activity is not surprising. The number of sulphydryl groups estimated by chemical means agrees with the values obtained by amino acid analysis. The higher value of 1.82 obtained using DTNB after performic acid oxidation in comparison with that of 1.29 obtained with BDC-OH would imply that the enzyme possesses either one disulphide bond and one free cysteine, or that it contains just two cysteines, one of which has buried residues and is unreactive with BDC-OH. The high content of proline is suggestive of little helical structure for the S. griseus neuraminidase. The amino-terminal amino acid was easily detected as alanine by the dansyl chloride procedure, and is probably exposed. The detection of only one dansylated amino acid further reflects on the purity of this enzyme preparation.

From the kinetic studies on the enzyme, it is apparent that the pH optimum (average 5.3) of S. griseus neuraminidase is dependent on the

substrate used. The shape of the pH curve is suggestive of the existence of ionisable groups in or close to the active site of the enzyme and the fact that the enzyme activity falls off on either side of the pH optimum would indicate that the active state of the enzyme is an intermediate form. On the question of pH, it is interesting that despite the reported differences in the catalytic pH optima of neuraminidases from different sources (Vibrio cholerae, Clostridium perfringens and Influenza virus) [33, 34], the optimum pH for adsorption to the affinity column of Cuatrecasas and Ilano [174] was about pH 5.5 for all the three different neuraminidases.

The K_m value of 1.8×10^{-4} M for sialyllactose obtained in the present study is quite close to the values reported for other neuraminidases [33, 34] but is slightly lower than the value of 4×10^{-4} M obtained by Kunitomo et al. [43] for the same enzyme. From the Dixon plot (Figure 2.16), NANA behaves as a competitive inhibitor of S. griseus neuraminidase, and in this respect this enzyme is similar to Vibrio cholerae and viral neuraminidase but different from the enzyme from Clostridium perfringens which is not significantly inhibited by NANA [33, 102]. The neuraminidase from an Arthrobacter species, described by Wang et al., [210] is also insensitive to inhibition by NANA. The K_i of NANA for S. griseus neuraminidase was about 4×10^{-3} M for MPN being about 20 times more than the K_m , and therefore making NANA a rather weak inhibitor of S. griseus neuraminidase.

Chapter 3

3. Mechanism of Neuraminidase Action

3.1 Introduction

"It is quite natural that enzyme chemists have, thus far, been occupied with the discovery of many kinds of enzymes, the ingenious methods of preparing them and the measurement of their activity. But at this point we must enquire into the chemical mechanisms by which they work".

L. Michaelis, 1946

The overall catalytic function of an enzyme may be attributed to a combination of the processes of substrate binding, the substrate specificity of the enzyme and the catalytic process itself. Although many compounds may bind to an enzyme, only certain types (substrates) lead to subsequent chemical reaction, while other types may affect the overall rate of an enzymic reaction in a positive (activatory) or negative (inhibitory) way. Generally, the molecular mechanisms which define the binding, specificity or catalytic activity of an enzyme depend on the enzyme's three-dimensional structure in solution. The crucial specific region on or near the enzyme surface encompassing all the amino acid chains that individually or in concert participate in binding the substrate to the enzyme and in performing the catalytic process has been termed the enzyme's 'active site'.

Alteration of these amino acid residues by physical or chemical means can contribute to an understanding of the chemical events by which they direct the catalytic function of an enzyme, and their identity constitutes a primary requirement for such an understanding. In addition, information on the structure and conformation of the active sites in an enzyme can be gained through studying the interaction of the enzyme with chemically modified substrates or inhibitors.

In the case of neuraminidase, important information regarding the action of the enzyme has been obtained through the modification of the sialic acid moiety, or the aglycone to which it is attached, in neuraminidase susceptible substrates. Examination of neuraminidase

substrates reveals four functional groupings: the carboxyl, the hydroxy chain, the acylated amino nitrogen and the aglycone (Figure 2.1)(page 28).

Schauer and Faillard [241] have reported that α -ketosides of 7-O-acetyl-8-O-acetyl and 7,8-O-diacetyl-N-acetyl neuraminic acid are cleaved by neuraminidase at a slower rate than the corresponding compounds with unsubstituted hydroxyl groups; whereas these results would indicate that a substitution of the hydroxyl groups in the polyhydroxy side chain of sialic acid is possible with only slight effect on the affinity of the enzyme for the substrate, the observed reduced inhibitory capacity of N-acetylneuraminic acid bearing bulky substituents in the polyhydroxy side chain (e.g. lipophilic cyclic acetals of N-acetylneuraminic acid : 7,9-O-benzylidene or 7,9-O-ethylidene derivatives of N-acetylneuraminic acid [242] (Figure 3.1) could possibly also be interpreted in terms of the size of the space in the enzyme available for the polyhydroxy chain of the substrate.

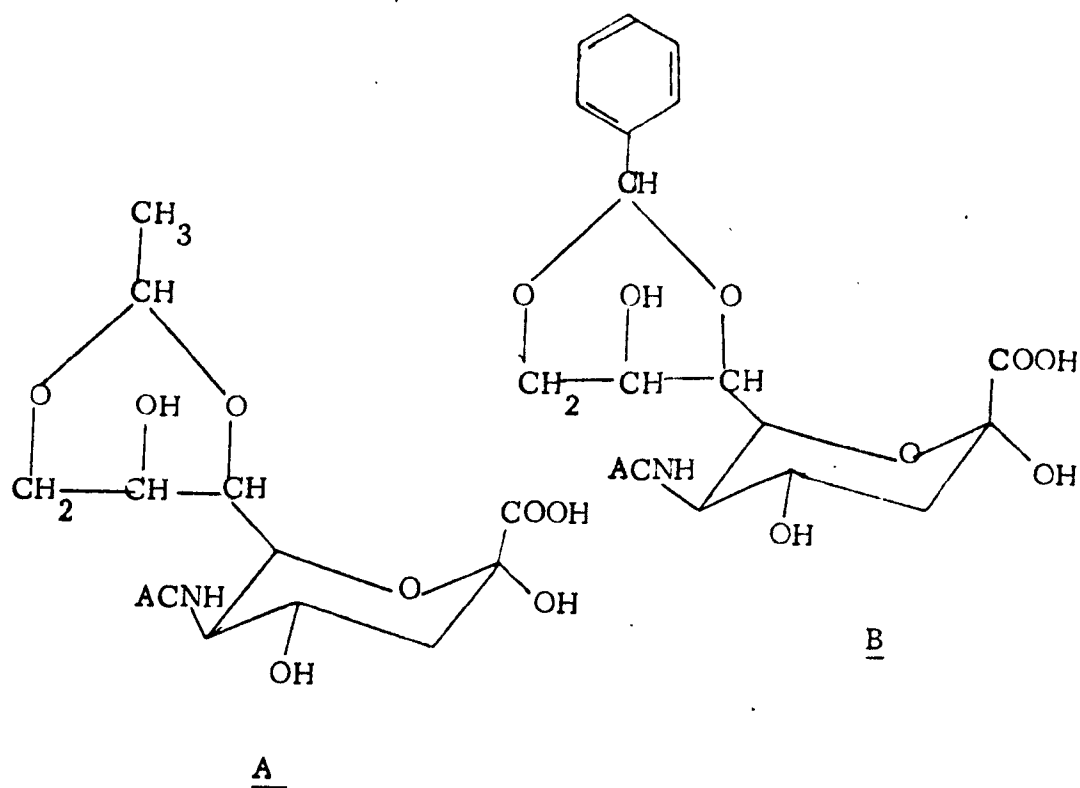


Fig. 3.1 A. 7,9-O-ethylidene-N-acetylneuraminic acid.
B. 7,9-O-benzylidene-N-acetylneuraminic acid.

Further, a negatively charged group (e.g. carboxyl) in the hydroxy chain of the substrate renders it resistant to neuraminidase [242], while the shortening of the hydroxy chain of sialic acid by periodate oxidation followed by borohydride reduction greatly reduces the susceptibility of the substrate to both bacterial and viral neuraminidase action [243]. N-acetyl-4-acetylneuraminic acid glycosides, such as are present in mucin, are resistant to bacterial neuraminidases [244].

The action of neuraminidase has been reported to vary with the nature of the N-substituent. The exchange of the N-acetyl ($\text{CH}_3\text{CO}-$) group in the substrate for an N-propionyl ($\text{CH}_3\text{CH}_2\text{CO}-$) N-formyl ($\text{HCO}-$) or N-glycolyl ($\text{CH}_2\text{OH}-\text{CO}-$) group reduces the rate of the enzymatic hydrolysis of the α -ketosidic bond, while replacement of the N-acetyl group with N-butyryl ($\text{CH}_3\text{CH}_2\text{CH}_2\text{CO}-$) group renders the α -ketosidic bond resistant to neuraminidase [242, 245]. These findings would suggest that neuraminidase possesses an active site pocket which the N-acetyl derivative substrate fits ideally and that an increase or decrease in the chain length of the acyl group tends to alter the critical distance and environment of the ketosidic bond from the catalytic groups in the active site of the enzyme.

Modification of the carboxyl group in the sialic moiety of neuraminidase substrates has also been shown to result in their reduced susceptibility to neuraminidase hydrolysis. Thus, esterification of the carboxyl group of sialic acid in these substrates or its transformation into primary alcohol or amido groups render the substrates resistant to neuraminidase and abolishes the inhibitory action of N-acetylneuraminic acid, even when the steric requirements for neuraminidase substrates are retained [242]. Colominic acid, the homopolymer of sialic acids in α -2 \rightarrow 8 ketosidic linkages, is usually a poor substrate for neuraminidase, releasing only 20% of its total sialic acid in one hour. When colominic acid is treated with alkali and then digested with neuraminidase, all the sialic acid is released in 1 hour. It is thought that some of the carboxyl groups of sialic acid are linked to the hydroxyl of the neighbouring monomer unit by an ester group to the C7 or C9 positions (Figure 3.2) [246].

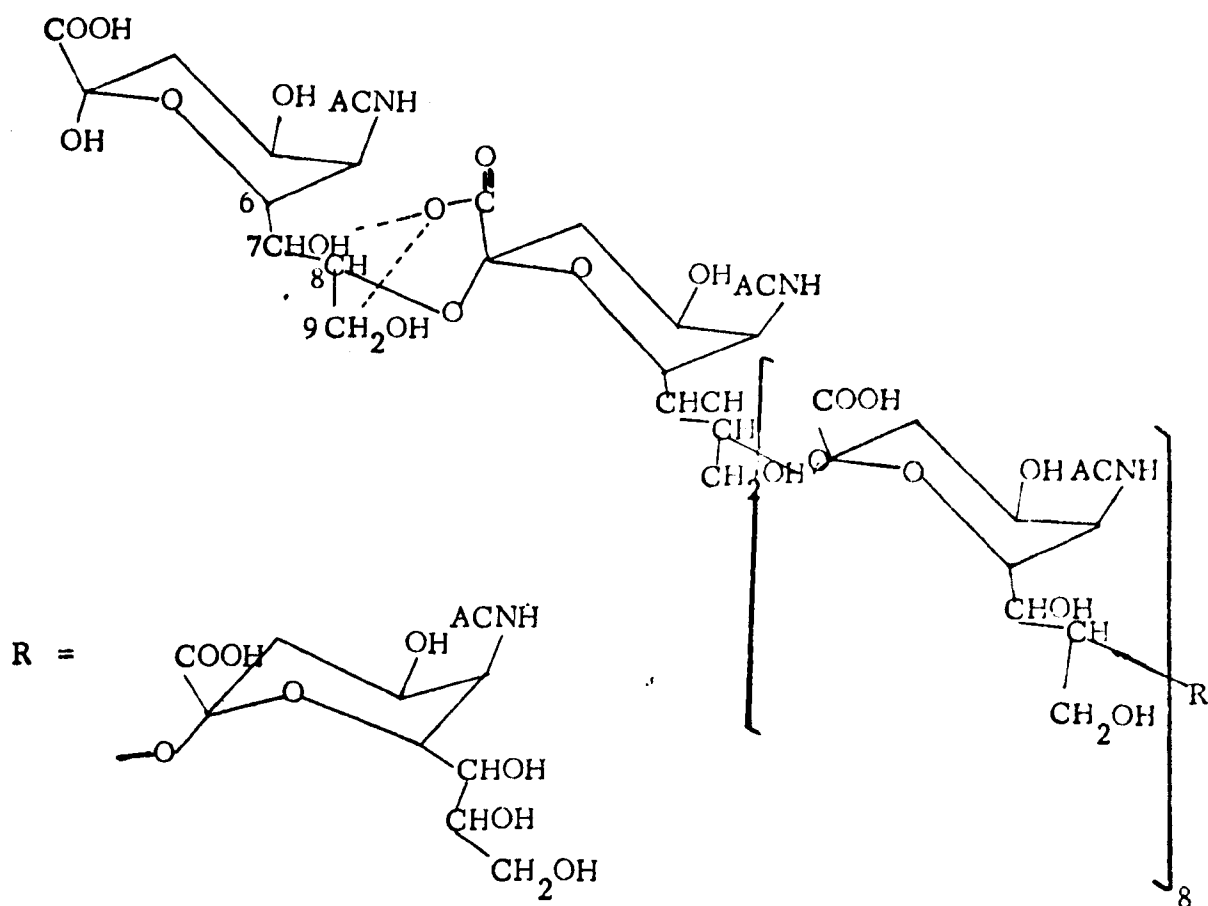


Fig. 3.2 Colominic acid showing the esterification of the carboxyl by C7 or C9 hydroxyl groups (-----)

Neuraminidases seem to require the carboxyl group of sialic acid in their substrates to be free and negatively charged for maximal activity [247] and evidence has been presented that the carboxyl group in the sialic acid moiety must bind to the enzyme in order to effect enzymatic cleavage of the ketosidic bond [247, 248].

As mentioned in Chapter 1, the aglycone in neuraminidase substrates may be a monosaccharide, oligosaccharide, glycoprotein, glycolipid, aliphatic or aromatic alcohol. Only slight variations in the ability of neuraminidases to hydrolyse α -ketosides of N-acetylneuraminic acid having different neutral aglycones have been found. It is interesting that α -ketosides containing neutral low molecular weight aglycones do not exhibit any affinity for the enzyme, an observation which reflects on the exolytic nature of neuraminidase. There are several cases in which the substitution of a bulky group in the aglycone results in diminished

activity of neuraminidase on such substrates, notably the resistance of monosialoganglioside to neuraminidase. After enzymatic removal of galactose or N-acetyl galactose, neuraminidase will release sialic acid from an otherwise resistant monosialoganglioside [245]. Also, in some glycoproteins, only 30-60% of sialic acid can be removed, although fragmentation of the macromolecules into small glycopeptides renders all sialic acid susceptible to neuraminidase [245]. Introduction of a positively charged aminogroup or incorporation of a carboxyl group in the aglycone of an α -ketoside of N-acetylneuraminic acid results in a substrate possessing low affinity for the enzyme [241, 245]. The aglycone may therefore be important to the action of neuraminidase in as far as its steric and electrostatic shielding of the ketosidic bond or its interaction with the essential carboxyl group of sialic acid is concerned.

Although the reactions catalysed by neuraminidase and the nature of the chemical structure required in the substrate in order to combine with the enzyme have been thoroughly investigated, little is known concerning the identity of those amino acids involved in substrate binding or catalysis. The question of the mechanism of neuraminidase has for the most part been approached, employing the philosophy of:

"Give me a man's food and I will tell you about the man".

L.M. Gerald, 1890

which is more like defining the structure of the ideal key for an existing lock. This approach suited the investigators who, although recognising the requirement for the key to possess a certain critical structure, did not study the mechanism by which the key turns in the lock, being more interested in the ideal fit or its prevention in as far as they were predominantly in search for a basis to design potent specific inhibitors of neuraminidase which would be protective against viruses containing this enzyme.

3.2 Chemical modification of amino acid residues in proteins

The recognition of the involvement of particular amino acid residues in the action of many enzymes has been facilitated by the availability of group-specific modifying reagents. Glazer et al. [249], Cohen [250]

and Stark [251] are a few of the many who have reviewed selected methods and analytical procedures employed in the modification of proteins, some of which methods have been adapted in the work reported in this thesis to assess the functional importance of various amino acid residues in the activity of neuraminidases. The reactivity of most proteins with these reagents arises from the reactivity of the side-chains of the basic amino acids (lysine, histidine and arginine), the acidic amino acids (glutamic and aspartic acids), the sulphur containing amino acids (cysteine and methionine) and the activated aromatic amino acids (tyrosine and tryptophan). The majority of protein modifications depend either on the nucleophilicity of the amino acid side-chains or on their ability to undergo oxidation.

In typical modification studies the enzyme is treated under appropriate conditions with a large excess of amino acid-modifying reagent or with one that can be maintained at a constant level; at various times aliquots are assayed for enzyme activity and for amino acid content. In practice, the reactivity on the enzyme surface may not correlate with the reactivity of the free amino acid in solution. Sometimes the decrease in activity associated with the modification reaction cannot be interpreted in a simple manner and often the reactivity of the protein residues cannot be consistently predicted or controlled. These limitations of the modification approach to studying the active site of an enzyme relate to the complex environment of the amino acid groups as defined by the protein structure. The term "group-specific", as applied to the modifying reagents, represents a level of optimism not often achieved in practice. In fact, some authors [250] have preferred the term "group-selective" as a more accurate description of the reality that successful chemical modification of a single type of functional group in total exclusion of other types is the fortunate exception rather than the rule. Some of the amino acids to be modified may be involved in maintaining the structure and some in the recognition and binding of substrates, and even if the modification of one group is accompanied by the inactivation of the enzyme, steric effects, charge and conformational effects must be discounted before the group modified can be assigned a role in the action of the enzyme.

Although chemical modification of proteins involves such complicated inter-related effects, a great deal of information has been obtained from the careful application of this technique to active site studies. By employing many different reagents, the effect of modifying various groups can often be judged from the correlation between the rate of inactivation of the enzyme. Further information has been obtained from modification studies conducted in the presence or absence of the enzyme's substrate or inhibitor. Also, in cases where side reactions are known to occur, there are often means of reversing the side reactions after the main modification reaction has been performed, or of preventing them altogether by employing certain specific conditions, especially of pH. It is also possible to assess the extent to which the dependence of activity on the conformational state of the protein is altered by the modification reaction by correlating its physical characteristics and its enzymatic activity.

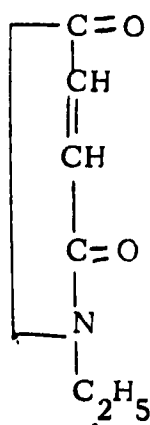
3.3 Active Site Studies on Neuraminidase

The results obtained from the interaction of the chemically defined substrates with neuraminidases and the observed absence of metal ion participation (except in the case Vibrio cholerae neuraminidase) in this interaction support the assumption that these enzymes possess catalytic sites of defined shape and size in which weak electrostatic forces and hydrogen-bonds are operative in the direct binding of the substrate. The kinetic investigations of the reactions of the pure enzymes with a range of substrates or inhibitors define the phenomena that the structure of these enzymes must explain. Structural investigation of the active site provides a rational framework in which the amino acid residues important in catalysis or specificity can be described; so far, chemical modification of the protein is the main approach by which such active groups may be discovered, and their functional role established.

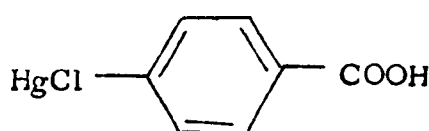
It is only recently that the approach of studying the active site of an enzyme by chemical modification has been applied to neuraminidase.

Until the studies of Hoyle [252] and Bachmayer [253], using group-specific reagents, insight into the nature of the active site of neuraminidase had been deduced from kinetic studies using various inhibitors, especially sulphydryl reagents, e.g. p-chloromercuribenzoate (PCMB).

The early work of Mohr [254] showed that such sulphydryl reagents had no effect on Vibrio cholerae neuraminidase, while in related studies Ada [255] reported inactivation of avian neuraminidase using PCMB and N-ethylmaleimide.



N-ethylmaleimide



p-chloromercuribenzoic acid
(PCMB)

Using these sulphydryl reagents, Tuppy and Palese [256] excluded the participation of sulphydryl groups in the activity of pig kidney neuraminidase. The neuraminidases of rat-liver and kidney have also been shown to lack sulphydryl function [257]. Kumimoto et al. [43] showed that neuraminidase from Cl. perfringens is inactivated by PCMB while the enzyme from S. griseus and Streptomyces purpeofuscus was not affected by treatment with this reagent.

From chemical modification studies of intact influenza virus, using iodine and fluorodinitrobenzene, Hoyle [252] excluded sulphydryl groups, lysine, arginine and tryptophan, but implicated tyrosine and histidine as part of the active site of viral neuraminidase. However, the results of Bachmayer [253] following oxidation of purified viral and bacterial

neuraminidases with N-bromosuccinimide suggested an essential role of tryptophan in either the active side of the enzyme or in maintaining the intact structure of the protein molecule required for activity of the enzyme. Further, nitration or acetylation of tyrosine residues and alkylation of cysteine residues affected neither the activity of influenza virus neuraminidase nor that of the neuraminidase from Cl. perfringens. In a recent study of neuraminidase from an Arthrobacter species [210], no inactivation was observed by reagents known to modify sulphhydryl, lysyl (only slightly affected enzyme activity when modified), carboxyl, histidiny and arginyl residues but treatment of the enzyme with N-bromosuccinimide resulted in complete inactivation.

It is yet to be established whether the apparent disagreement in the results of these studies reflect on the different mechanisms of action for neuraminidases of different origin or on the limitations of the techniques employed (for instance, side reactions, variations and interferences in enzyme activity determinations, effectiveness of the modification reactions, etc.) or on the differences in structures of these enzymes. The studies reported in this thesis were directed at comparing the effects of chemical modification of lysyl, arginyl, histidiny, carboxyl and tryptophan residues on the activity of neuraminidases from S. griseus and Cl. perfringens and influenza virus in the hope of identifying the residues essential in catalysis. Similar studies were carried out on the neuraminidase from Vibrio cholerae neuraminidase by Groundwater [258] in this laboratory.

3.4 Chemical Modification Reactions Employed

The experimental procedures for the chemical modification of neuraminidases using the reactions described in this section are outlined in section 3.5.

3.4.1 Modification of lysine residues

The reaction of 2, 4, 6-trinitrobenzene sulphonic acid (TNBS) which under alkaline conditions appears to be predominantly restricted to α - and ϵ -amino groups (Figure 3.3) was used to

modify lysine residues in neuraminidase.

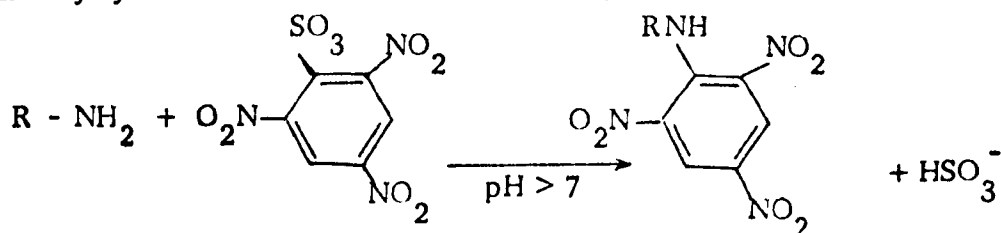
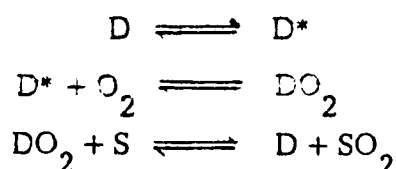


Figure 3.3 Reaction of TNBS with amino groups in proteins [259]

3.4.2 Modification of histidine residues

Photooxidation of proteins in solution by irradiation is conducted in the presence of oxygen and a photooxidising dye such as Rose Bengal. After absorption of light of suitable wavelength, the dye becomes energised, and is able to transfer energy directly to the photo-oxidisable residue oxidation. The mechanism of photochemical oxidation is complex and variable; it is thought to proceed viz: [260]



where D represents the dye, D* an excited electronic state of the dye and S the substrate (such as histidine). Although a variety of functional amino acids other than histidine, e.g. methionine, cysteine, tryptophan and tyrosine are subject to photooxidation, a degree of selectivity can be achieved by controlling the pH of the reaction [261]. Thus in acidic media methionine, cysteine, tryptophan and sulphydryl groups are oxidised while at neutral pH histidine is the most susceptible to oxidation. Diethyl pyrocarbonate has found some favour in its specific reaction with histidine residues in proteins (Figure 3.4) at slightly acid pH [262].

3.4.3 Modification of arginine residues

A variety of procedures are available for the modification of arginine residues in proteins [249, 250, 251, 263]. The majority of arginine modification procedures have been conducted commonly employing 1,2-dicarbonyl compounds such as phenyl glyoxal or

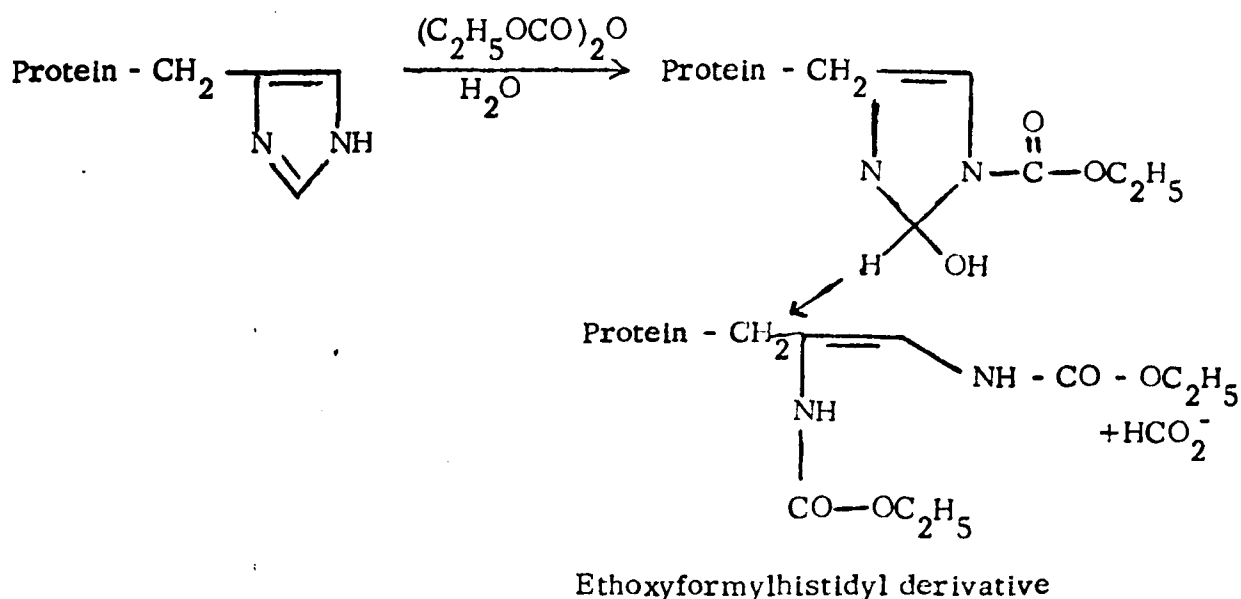
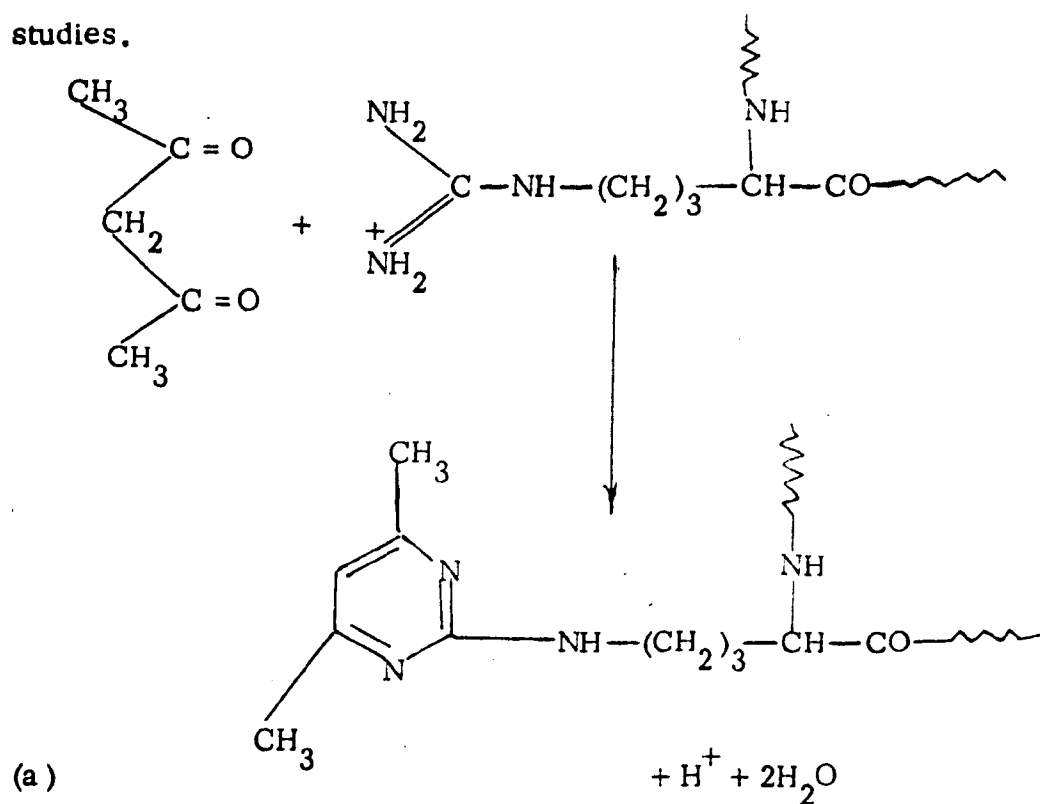


Figure 3.4 Modification of histidine with diethyl pyrocarbonate

butanedione and the recently described 1,3-dicarbonyl reagent (2,4-pentanedione) [264]. Although these procedures yield products which have not yet been fully characterised, they are performed under mild conditions and have been widely employed in enzyme studies.



(continued on next page)

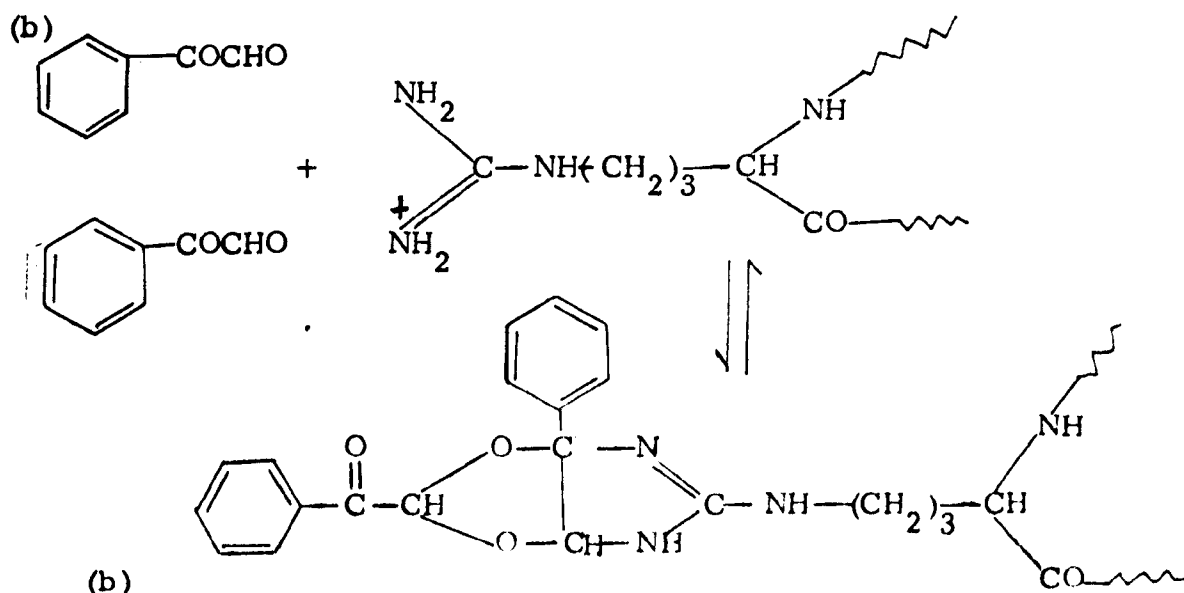


Fig. 3.5 Reaction of arginyl side-chains in proteins with (a) 2,4-pentanedione [264]. (b) phenylglyoxal [265]

The reaction of 2,4-pentanedione with proteins effects the modification of both arginine and lysine residues. However, the modified lysine residues can be regenerated by dialysis or by treatment with hydroxylamine (Figure 3.6).

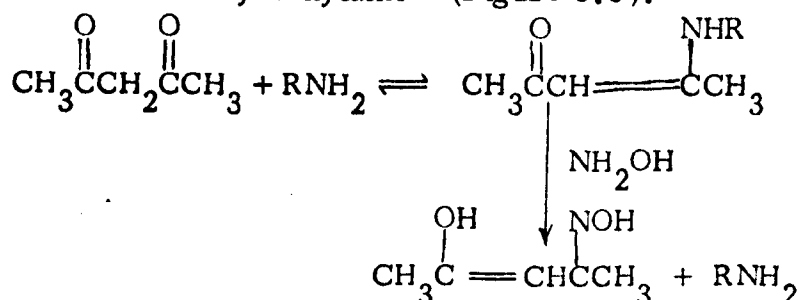


Fig. 3.6 Regeneration of 2,4-pentanedione-modified lysines (enamines) by reaction with hydroxylamine

The reaction of phenylglyoxal with proteins at pH 8 is mainly restricted to arginine residues, although 2-amino groups may also react.

3.4.4 Modification of carboxyl groups

The popular method of chemically modifying carboxyl groups in proteins involves the activation of the carboxyl groups by a water-soluble carbodiimide such as 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and the subsequent reaction of the activated carboxyls with a nucleophile, e.g. glycine methyl ester [266] at slightly acid pH.

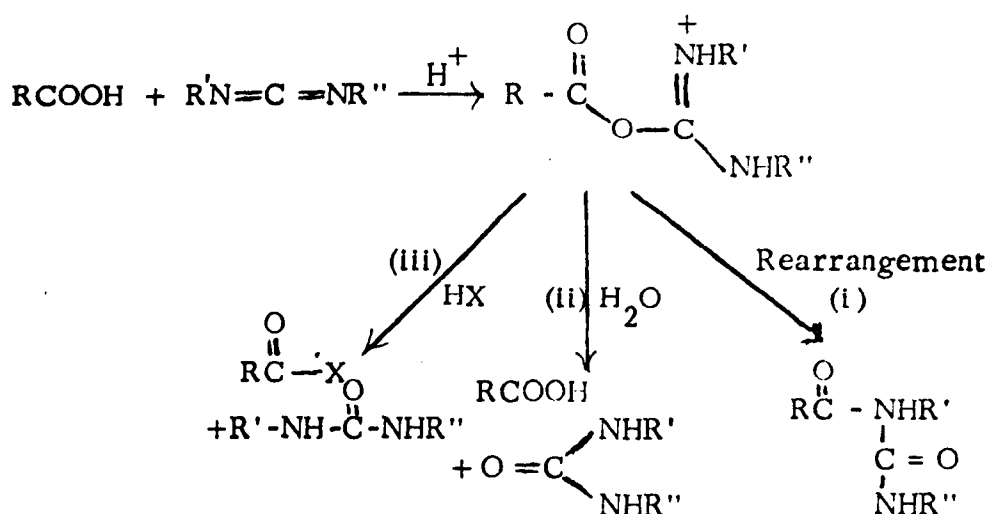
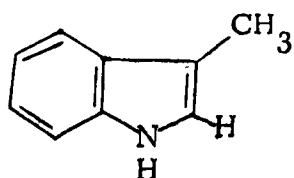
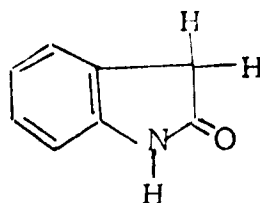


Fig. 3.7 Reaction of a water-soluble carbodiimide ($\text{R}'\text{N}=\text{C}=\text{NR}''$) with a protein carboxyl group (RCOOH) followed by rearrangement (i), hydrolysis (ii) or attack by a nucleophile (HX) to form the modified carboxyl group (RCOX) (iii)

Carbodiimides react with a number of functional groups at acid pH, the predominant of which are carboxyls, sulphhydryls and tyrosines. The carbodiimide modified tyrosine residues may be regenerated by reaction with hydroxyl-amine, but no similar treatment has been reported to regenerate modified sulphhydryl groups. Exposure of proteins to carbodiimide during modification studies leads to a covalent polymerisation of some protein molecules even in dilute acidic solutions [267]; however, nucleophilic addition reactions are less susceptible to polymerisation effects.

3.4.5 Modification of tryptophan residues

Modification of tryptophan residues in proteins is often effected by oxidation with positive halogen reagents, particularly N-bromosuccinimide (NBS). The oxidation is conducted in an acidic medium with carefully controlled amounts of NBS when the indole moiety of tryptophan (A) is transformed to an oxyindole (B) accompanied by a reduction in absorption at 280 nm [268].

AB

Side reactions encountered in the reaction of NBS with proteins may include the oxidation of tyrosine, methionine, cysteine, histidine or arginine residues as well as peptide cleavage.

3.5 Chemical Modification of Neuraminidases

Experimental Procedure

3.5.1 Preparation of neuraminidases

S. griseus neuraminidase was prepared and purified as described in Chapter 2. Clostridium perfringens neuraminidase was obtained commercially and further purified by affinity chromatography according to the method of Brossmer et al. [204], using N-(4-nitro-phenyl-oxamic)acid with Sepharose 4B containing 1,6-diaminohexane as spacer group. Viral neuraminidase was isolated from influenza (A/Japan/305/57) virus. The virus was grown in the allantoic sac of 11-day-old chick embryos and was purified and concentrated from virus-infected allantoic fluid by low and high speed centrifugation essentially as described previously [271]. The virus preparation thus obtained was used to prepare neuraminidase by incubation with nagarse followed by sedimentation on linear sucrose gradients as described by Kendal and Eckert [211]. The amount of neuraminidase was determined based on its enzyme activity when assayed on fetuin as described below. A neuraminidase preparation (4.5 ml) of specific activity 285 U/ml was obtained from 1 litre of virus-infected allantoic fluid.

3.5.2 Enzyme assays

Unless otherwise stated, neuraminidase activity was determined using fetuin (5.0 mg/ml in assay buffer) as the substrate. The assay buffer was 0.05 M sodium acetate, pH 5.3, in the case of S. griseus neuraminidase, 0.05 M sodium acetate, pH 4.5, for Cl. perfringens neuraminidase and 0.1 M phosphate buffer, pH 6, in the case of

influenza virus neuraminidase. The enzyme solution (in amounts shown in the text) was added to the fetuin solution (0.5 ml) and the mixture (total volume 1.0 ml, made up with assay buffer) was incubated at 37° C for 60 minutes, and the released sialic acid was determined as described earlier.

3.5.3 Lysine Modification

TNBS (trihydrate), obtained commercially, was recrystallised from hydrochloric acid according to the method described by Fields [259] before use. The incubation mixtures in the lysine modification experiments contained neuraminidase (amount depending on the source of the enzyme, see legend, Figure 3.8) in 0.1 M sodium phosphate buffer, pH 7 (2.0 ml) containing 5 mM TNBS. In the control incubation mixtures, TNBS was omitted. The reactions were carried out at room temperature for 6 hours during which time aliquots (200 µl) were withdrawn at intervals and passed through a Sephadex G-25 column (1 x 10 cm), equilibrated and eluted with the appropriate assay buffer. The effluent fractions contained mainly in the void volume were assayed for neuraminidase activity and for protein content. It was not possible to detect protein in significant amounts in effluent fractions of the viral enzyme. However, on account of its high molecular weight, its total exclusion from the Sephadex G-25 column was assumed and the volume of the effluent fraction containing the enzyme was measured and compared with sample loaded on the column (200 µl) to obtain the dilution of the original enzyme solution.

From the results presented in Figure 3.8, it is apparent that the modification of lysine residues with TNBS under the conditions described does not appreciably affect the activity of the three neuraminidases. Viral neuraminidase loses about 20% of its original (control activity), Cl. perfringens neuraminidase loses only 10% and S. griseus neuraminidase loses 5% of its original activity.

3.5.4 Histidine modification

Histidine residues in viral, S. griseus and Cl. perfringens neuraminidases were modified essentially according to the procedures described in similar studies by Dickenson and Dickinson [269].

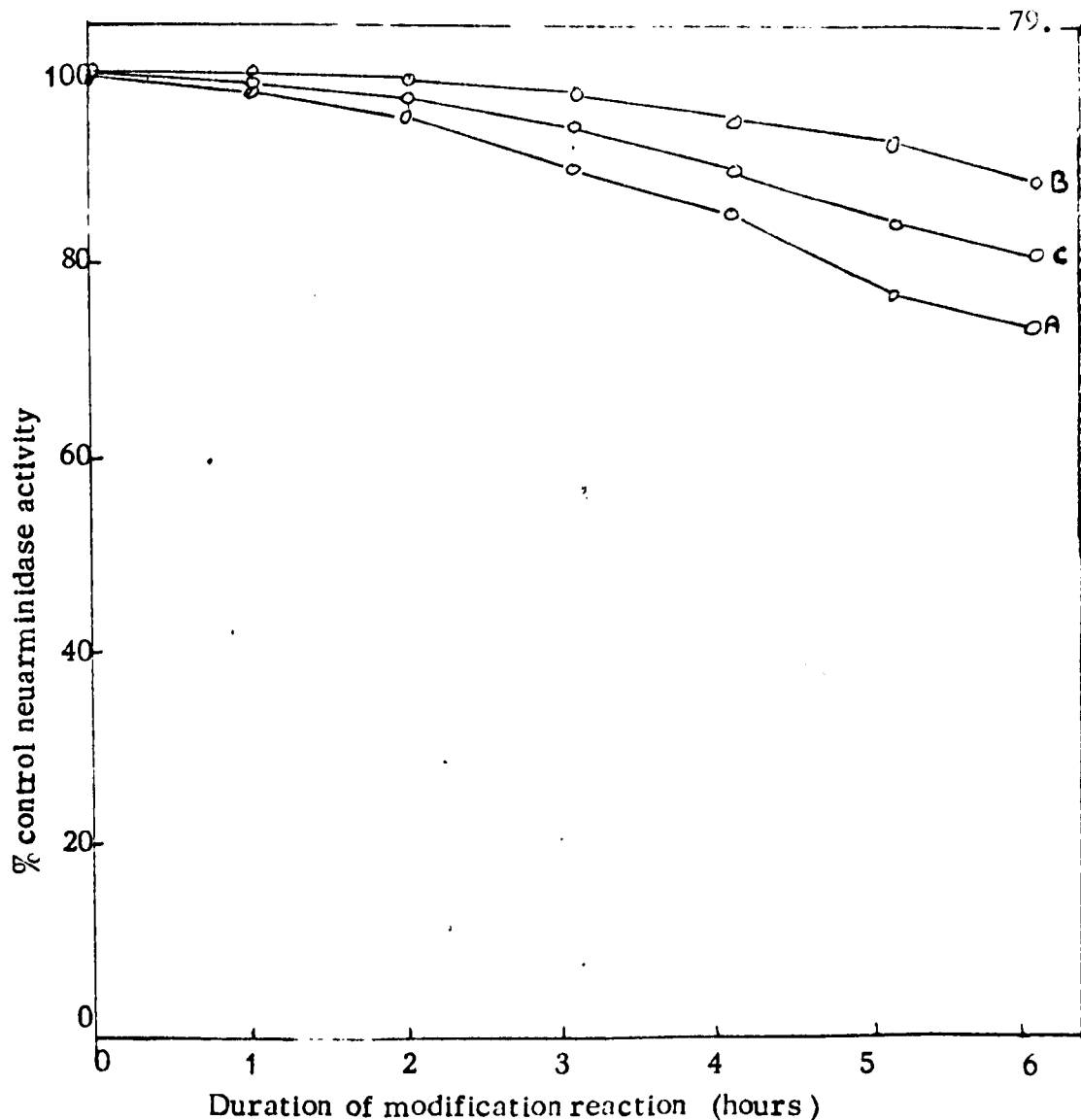


Fig. 3.8 Effect of lysine modification on the activity of neuraminidases. A. viral neuraminidase (30 units). B. S. griseus (0.3 mg). C. Cl. perfringens neuraminidase (0.4 mg).

Photooxidation catalysed by Rose Bengal was performed at 25⁰ C in the dark. The reaction mixtures, each in a total volume of 3.0 ml (0.1 M sodium phosphate buffer, pH 7), contained neuraminidase (S. griseus neuraminidase (0.3 mg), Cl. perfringens neuraminidase (0.4 mg) and viral neuraminidase (30 units)) and Rose Bengal (1.0 μ M) in a cuvette held before a 500 W tungsten lamp. To counteract the heat dissipated by the lamp, ice-cold water was passed around the surfaces of the cuvette. Oxygen was bubbled gently through the contents of the cuvette. The oxidation was carried out for 60 minutes during which time 200 μ l aliquots were removed at intervals and

passed through a Sephadex G-25 column (1 x 10 cm), equilibrated and eluted with the appropriate assay buffer before determining their protein and neuraminidase content.

The control reaction mixtures consisted of the enzyme and Rose Bengal in the quantities employed in the test samples, but were incubated at 25^o C in total darkness. A similar procedure (for the test and control reactions) was followed employing neuraminidase solutions containing 0.05 M NANA or 0.2 mg/ml sialyllactose.*

Modification of histidine residues in each of the three neuraminidases was also carried out by incubating neuraminidase (S. griseus neuraminidase, 0.2 mg; Cl. perfringens neuraminidase, 0.4 mg; viral neuraminidase (30 units) with and without (1.5 mM) diethyl pyrocarbonate in 0.1 M sodium phosphate buffer, pH 7.0 (total volume, 1.0 ml) for 90 minutes at room temperature. During this period the reaction mixtures were gently stirred, and at intervals aliquots of the reaction mixture (100 µl) were removed and subjected to gel filtration on a Sephadex G-25 column (1 x 10 cm), previously equilibrated with the appropriate assay buffer before their protein and neuraminidase contents were determined. The results obtained in these studies are represented in Figure 3.9.

3.5.5 Arginine modification

(i) 2,4-pentanedione

Neuraminidase (amounts as in legend, Figure 3.10) in 0.5 M sodium phosphate buffer, pH 8.6 (2.0 ml) was mixed with 2,4-pentanedione (distilled before use) added to a total concentration of 50 mM. At various time intervals, an aliquot (200 µl) of the reaction mixture was withdrawn and subjected to gel filtration on a Sephadex G-25 column (1 x 10 cm), using the appropriate assay buffer as the eluent. A fraction of the modified enzyme was assayed for activity and for protein and to the remainder hydroxylamine was added to a final concentration of 0.2 M. After 2 hours at 37^o C, the solution was passed through a Sephadex G-25 column eluted with the assay buffer and the protein content and neuraminidase activity of the effluent were determined using the assay procedure described earlier.

* NANA was used with S. griseus and viral neuraminidases while sialyllactose was used when treating Cl. perfringens neuraminidase.

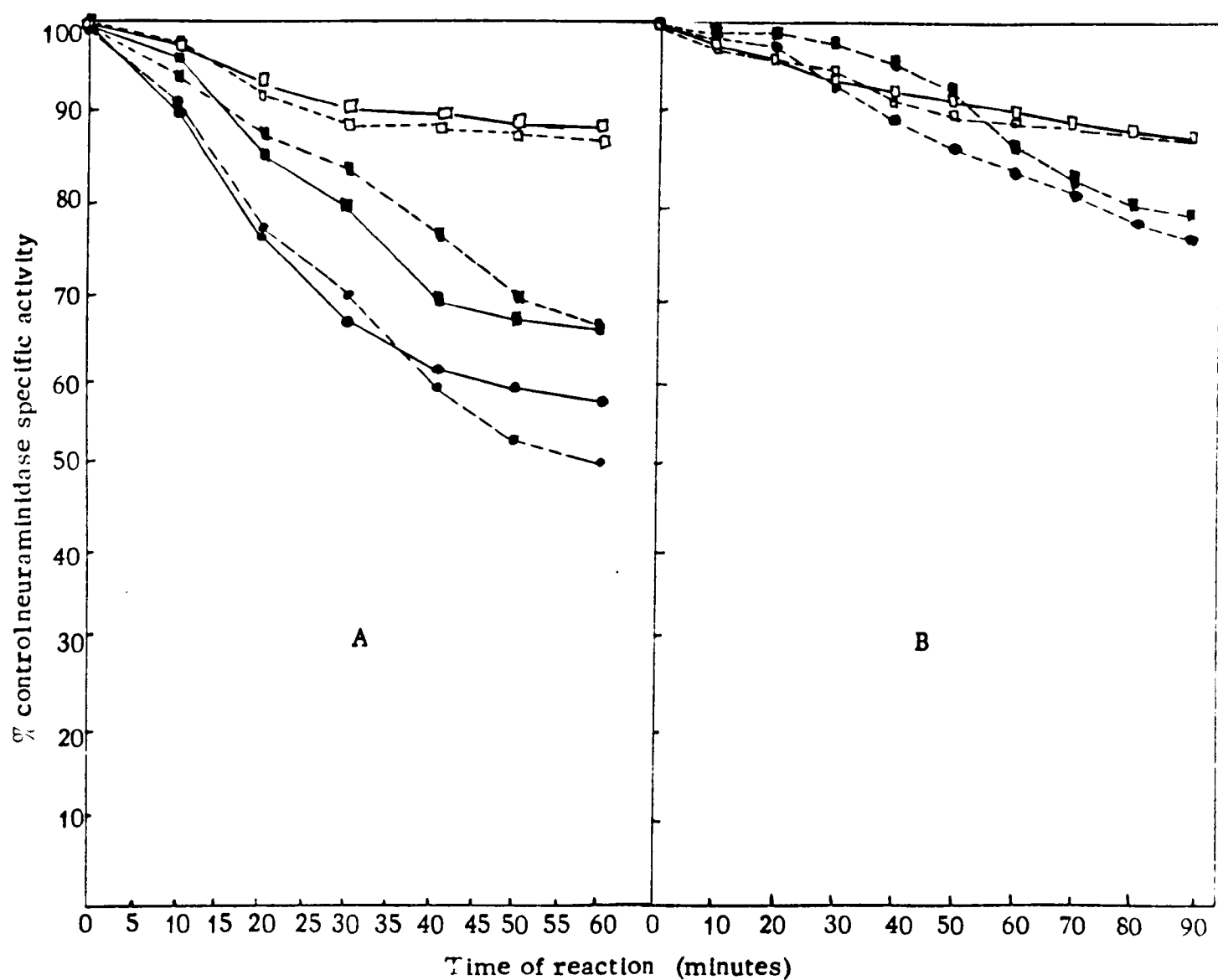


Fig. 3.9 Modification of histidine residues in neuraminidase of *S. griseus* (□), *Cl. perfringens* (■) and influenza virus (●) by photooxidation (A) and by reaction with diethyl pyrocarbonate (B) in the presence (solid lines) or absence of 0.05 mM N-acetylneuraminic acid (broken lines).

* In the case of *Cl. perfringens* neuraminidase, 0.2 mg/ml sialyllactose was used instead of 0.05 M NANA (solid lines), without sialyllactose (broken lines).

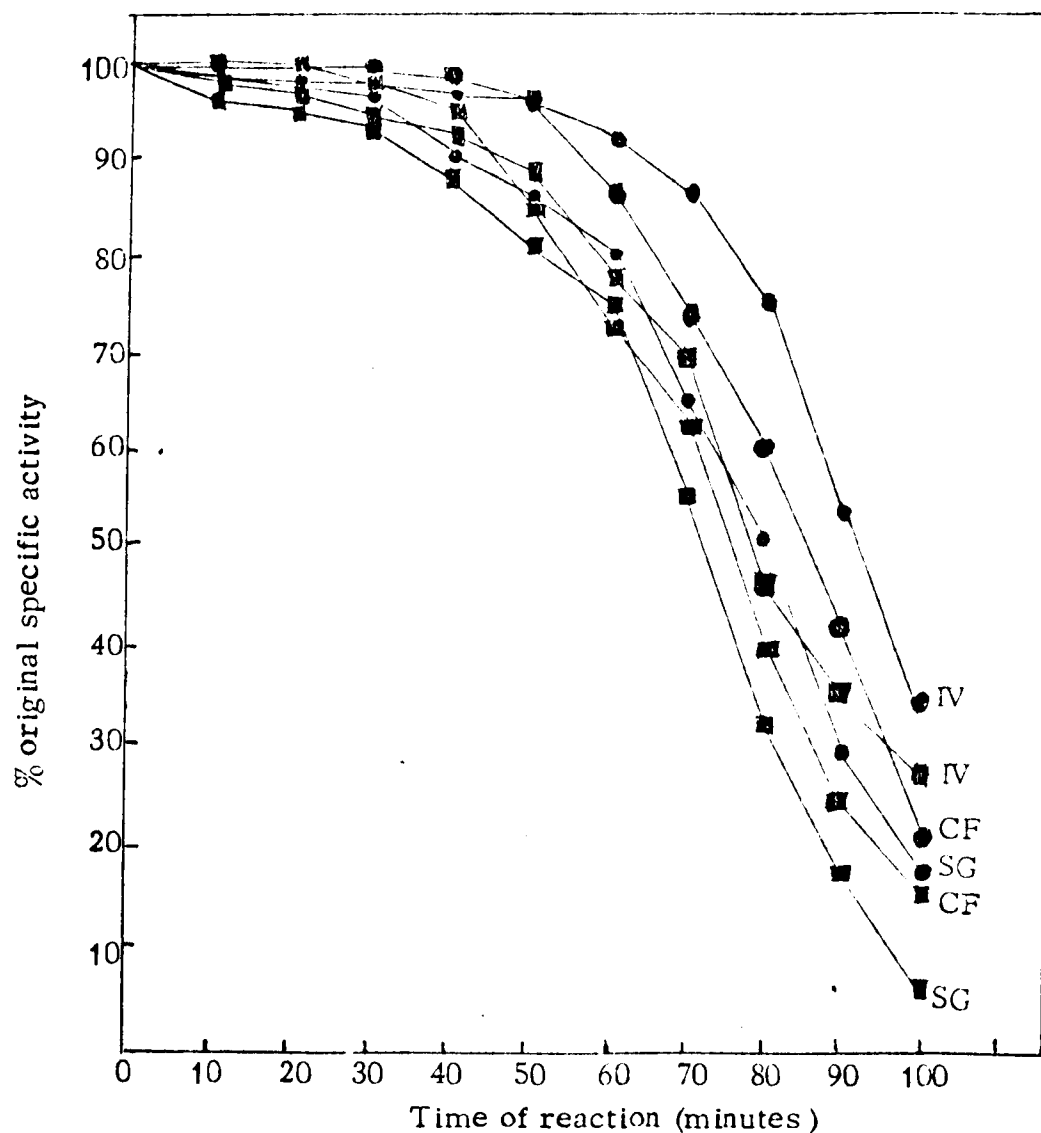


Fig. 3.10 Effect of modifying arginyl residues with 2,4-pentanedione on the activity of neuraminidase from *S. griseus* (SG), *Cl. perfringens* (CP) and influenza virus (IV); enzyme activity (—■—■) before, (—●—●) after treatment with hydroxylamine. Amounts of enzymes modified: SG (0.3 mg), CP (0.4 mg) and IV (40 units). The control enzyme activity was obtained by assaying the enzyme solution kept under identical conditions but in the absence of the modifying reagent.

(ii) Protection of *S. griseus* and viral neuraminidases with NANA

S. griseus neuraminidase (0.2 mg) was incubated in 0.5 M sodium phosphate buffer, pH 8.6 (2 ml) with N-acetylneuraminic acid (30 mM) for 30 minutes before being treated with 2,4-pentadione (40 mM) and hydroxylamine as described above. Figure 3.11 shows the results obtained. A similar procedure was followed using viral neuraminidase (40 units).

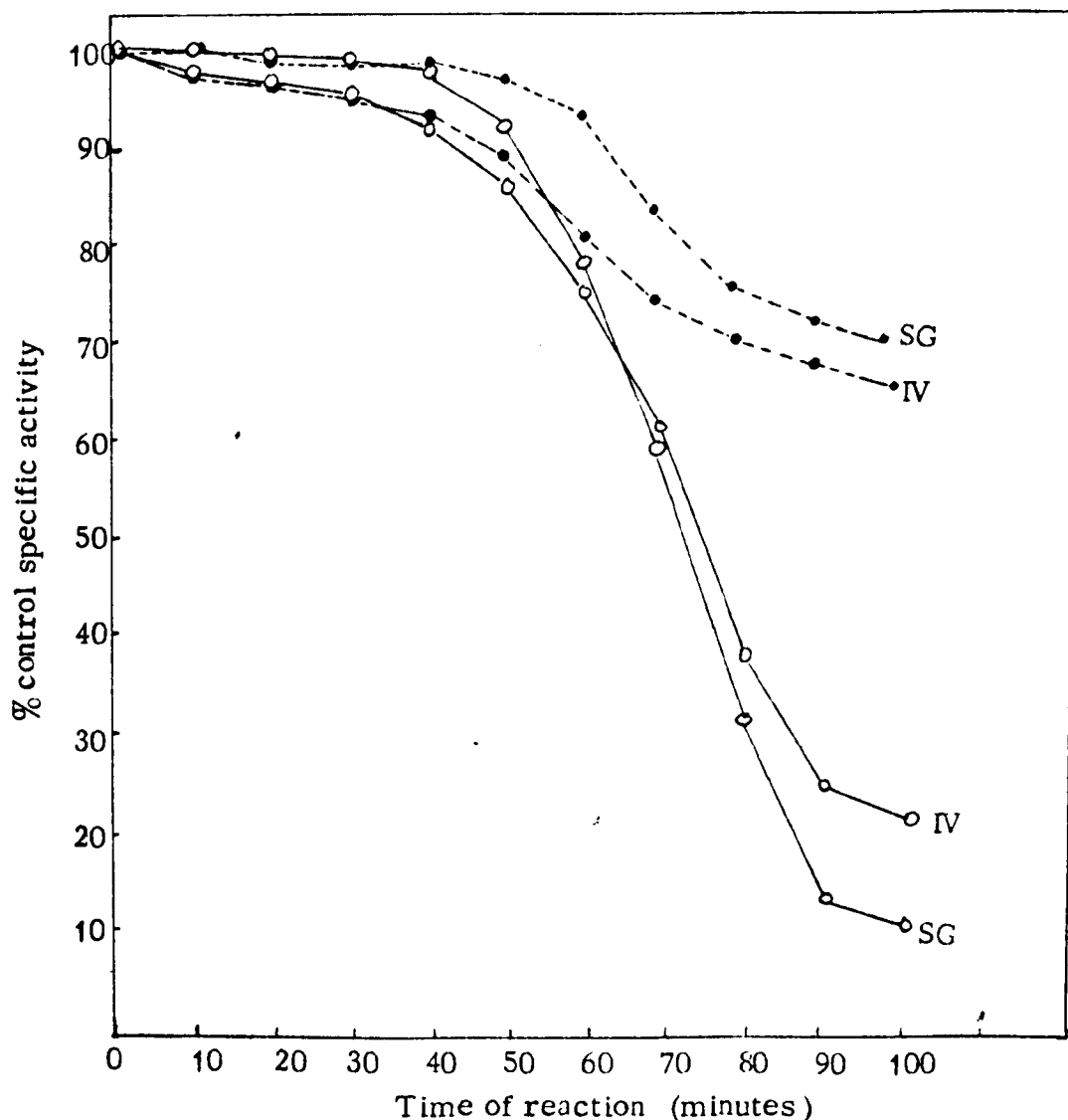


Fig. 3.11 Protection of *S. griseus* (SG) and viral (IV) neuraminidases against arginine modification by NANA.

(—○—○—) unprotected, (---●---●---) protected enzyme.

(iii) Phenylglyoxal

Modification reactions with phenylglyoxal were carried out on *S. griseus* and *Cl. perfringens* neuraminidases at room temperature (22°C) in 0.1 M N-ethylmorpholine acetate buffer, pH 8.0 [265]. The exact concentrations of the enzyme and reagent are given in the figure legends. The reactions were initiated by mixing the reagent and enzyme in a total volume of 1.0 ml. When the substrate (sialyllactose) or inhibitor (NANA) were used in protection experiments, they were incubated with the enzyme solution before the modifying reagent was added. In these experiments, a 200 μ l

aliquot of the reaction mixture was removed at various time intervals and the reaction was stopped by passage of this aliquot through a Sephadex G-25 (1 x 10 cm, equilibrated and eluted with the appropriate assay buffer at 4° C) to remove excess reagent. The effluent fractions containing protein were pooled and assayed for enzyme activity. The control incubation mixtures, which were similarly treated, contained the same amount of the enzyme as in the test, but lacked the modifying reagent.

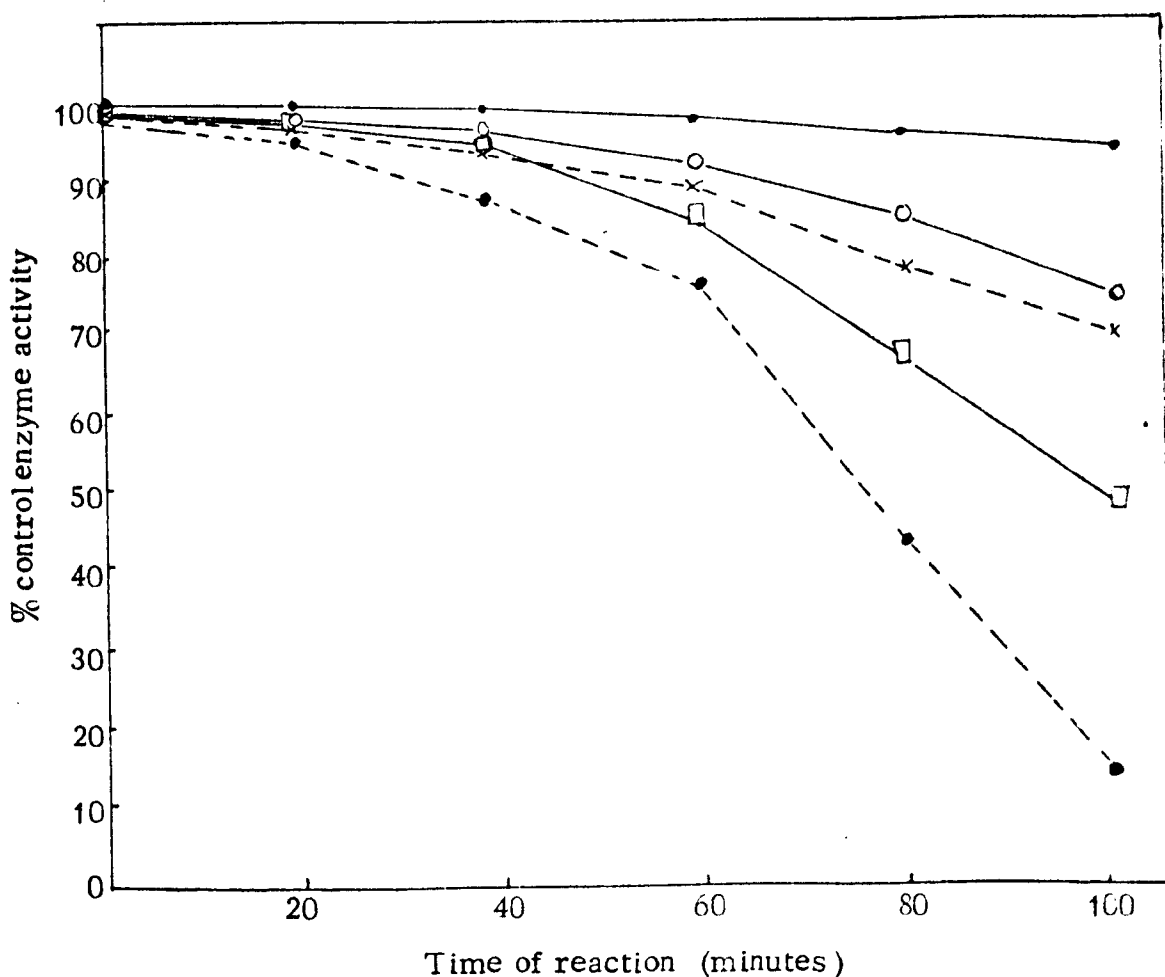


Fig. 3.12 Reaction of phenylglyoxal with *S. griseus* neuraminidase. The reaction was followed by the loss of enzyme activity. The modification reaction was carried out at 22° C in 0.1 M N-ethylmorpholine acetate buffer, pH 8.0 (1.0 ml) containing 0.5 mg neuraminidase. The residual enzyme activity determined after the gel filtration step was compared with that of the control (100% enzyme activity). The phenylglyoxal concentrations in different runs were: 0 (—●—●), 1.0 mM (—○—○), 2.5 mM (—□—□) and 5.0 mM (—●-●-). —x-x-x-x- modification of arginine residues in the presence of 50 mM NANA using phenylglyoxal at 5 mM concentration.

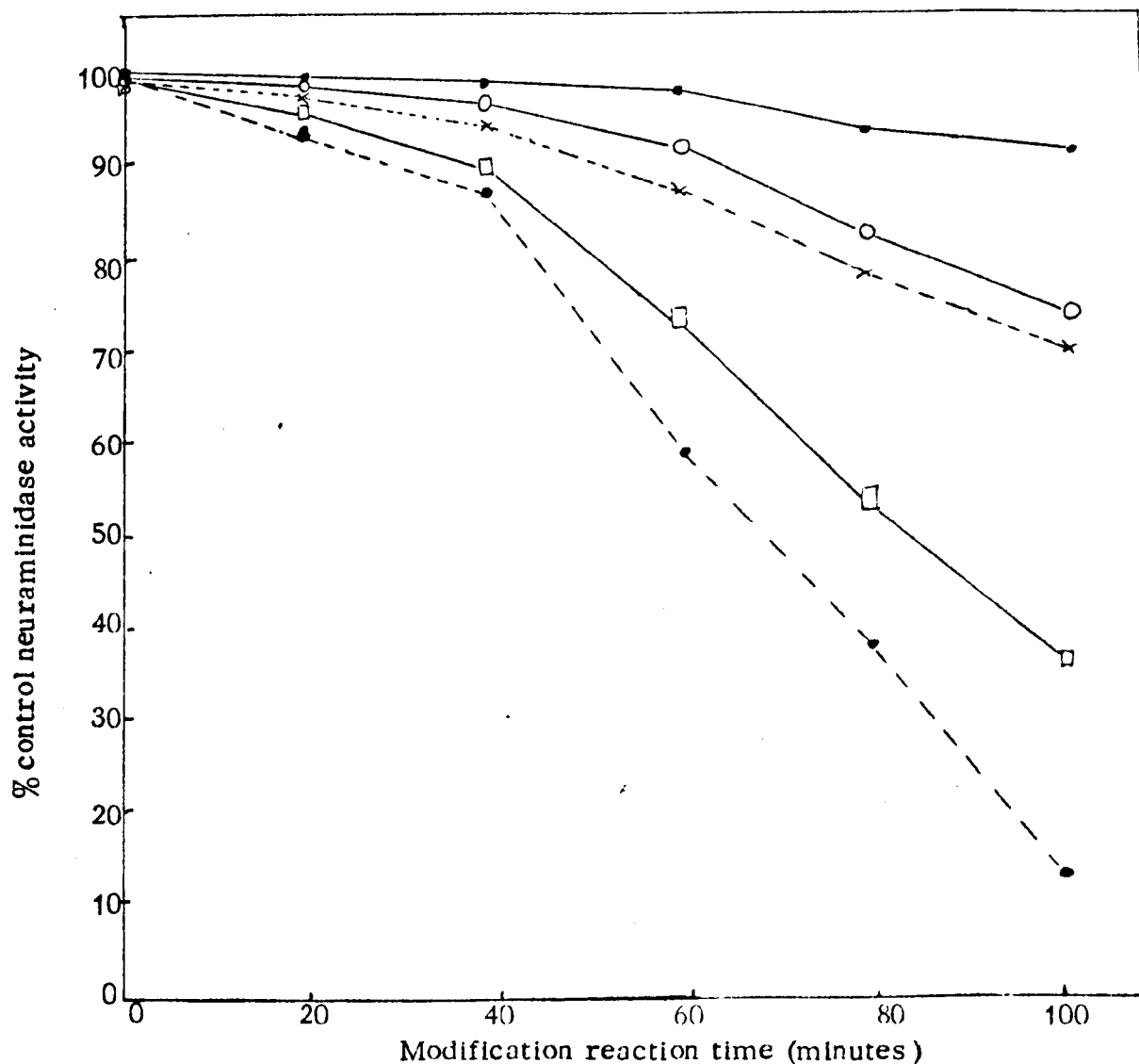


Fig. 3.13 Reaction of phenylglyoxal with *Cl. perfringens* neuraminidase.

Reaction conditions and measurement of modification as described for *S. griseus* neuraminidase. The enzyme concentration employed was 0.1 mg/ml and the phenylglyoxal concentrations in the various runs were 0 (●—●), 1.5 mM (○- -○), 2.5 mM (□—□) and 5.0 mM (×- -×) in the presence of 0.1 mg/ml sialyllactose.

(iv) Incorporation of [7-¹⁴C]phenylglyoxal* in *S. griseus* neuraminidase

The total number of arginine residues modified was determined by incorporation of [¹⁴C] phenylglyoxal; the number of arginine residues involved in the active site of *S. griseus* neuraminidase was determined by reacting the enzyme with unlabelled phenylglyoxal in the presence of NANA and after removal of excess reagent and NANA by gel

* [¹⁴C] phenylglyoxal was a gift of Dr. V.W. Armstrong, Max-Planck Institut, Gottingen. Its homogeneity was checked by comparing it with commercially available, unlabelled phenylglyoxal of known purity by thin layer chromatography. The [¹⁴C] phenylglyoxal hydrate had a specific activity of 1350 cpm/nmole and was used without dilution with unlabelled phenylglyoxal.

filtration, the modified enzyme was further reacted with [^{14}C] phenylglyoxal and from the label incorporated, the number of arginine residues protected by NANA were calculated, assuming a stoichiometry of 2 phenylglyoxal molecules per arginine residue modified [265].

The incorporation of [^{14}C] phenylglyoxal into *S. griseus* neuraminidase was determined by incubating the enzyme (0.5 mg) at 37°C in a final volume of 1.0 ml with [^{14}C] phenylglyoxal (5 mM) in 0.1 M N-ethylmorpholine acetate buffer, pH 8.0, for 3 hours. After this period, the incubation mixture was mixed with 10% trichloroacetic acid (2.0 ml). The resulting mixture was then filtered over a nitrocellulose membrane filter (pore size $0.45\ \mu$, 25 mm diameter). The filter was washed with 20 ml 10% trichloroacetic acid, dried under an infra-red lamp. The filter was placed in a scintillation vial containing 10 ml scintillant (Triton, butyl B.P.D.) and the radioactivity determined in a Packard Tri-carb liquid scintillation counter.

S. griseus neuraminidase (0.5 mg) was also incubated at 37°C in a final volume of 1.0 ml in 0.1 M N-ethylmorpholine acetate buffer, pH 8.0, containing NANA (30 mM) with unlabelled phenylglyoxal (2.0 mM) for 2 hours at room temperature. The reaction mixture was then passed through a Sephadex G-25 column and eluted with 0.1 M N-ethylmorpholine acetate buffer, pH 8.0. The effluent fractions containing protein (as assayed according to the method of Lowry [215]) were pooled and reacted with [^{14}C] phenylglyoxal (5.0 mM) for 2 hours. The resulting reaction mixture was then mixed with 10% trichloroacetic acid and further treated as described above to determine the radioactivity incorporated. Table 3.1 summarises the results obtained.

Table 3.1 Incorporation of [^{14}C] phenylglyoxal in *S. griseus*
neuraminidase

Reaction	Amount enzyme modified (μg)	Radioactivity [^{14}C] PG* incorporated (cpm)	No. of arginine residues labelled/** 32,000 m.wt.
Neuraminidase + [^{14}C] PG	500	342180	8.1
Neuraminidase + NANA + PG (unlabelled) + [^{14}C] PG	500	54849	1.3

* PG - phenylglyoxal

** The number of arginine residues labelled were calculated using a stoichiometry of 2 phenylglyoxal molecules per arginine residue modified [265] and the specific activity of [^{14}C] phenylglyoxal of 1350×10^3 cpm/ μmole . A molecular weight of 32,000 for *S. griseus* was assumed.

Thus for the total modification with [^{14}C] phenylglyoxal, the 342180 cpm obtained correspond to

$$\frac{342180}{1350 \times 10^3} \mu\text{moles of phenylglyoxal}$$

$$\text{and to } \frac{342180}{1350 \times 10^3 \times 2} \mu\text{moles of arginine residues}$$

$$\text{labelled in } \frac{500}{32000} \mu\text{moles of neuraminidase.}$$

\therefore 1 μmole of neuraminidase contains

$$\frac{342180 \times 32000}{1350 \times 10^3 \times 2 \times 500} \mu\text{moles of labelled arginine residues}$$

= 8.1 labelled arginine residues.

The value of 3-3.5 arginine residues obtained in an earlier report (see Appendix V, Kabayo, J.P. and Hutchinson D.W., FEBS Letts., 78, 223) for the same enzyme disagrees with the value of 8.1 calculated above. The value of 3-3.5 was obtained after reacting 3.7 mM phenylglyoxal with neuraminidase for 120 minutes while the value of 8.1 was obtained after a more thorough treatment of the enzyme with 5 mM phenylglyoxal for 3 hours.

3.5.6 Carboxyl modification

(a) The procedure described by Carraway and Koshland [266] was used to modify carboxyl groups in *S. griseus*, *Cl. perfringens* and influenza virus neuraminidases. In the modification reactions, a solution at pH 4.75 containing neuraminidase (amounts shown in Figure 3.14 legend) in 1.0 ml and glycine ethyl ester hydrochloride (1.33 M) was kept at 25°C in a water-jacketed vessel attached to a pH-stat. Solid 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was added to a final concentration of 0.5 M to initiate the reaction. The pH of the reaction mixture was kept at pH 4.75 by automatic titration with 1 N HCl and aliquots (200 µl) were removed periodically and subjected to gel filtration on Sephadex G-25 (as described in the other types of modifications above, except that the columns were equilibrated and eluted with 0.05 M sodium phosphate buffer, pH 6, instead of acetate buffer [266]) before the specific enzyme activities were determined. In the control reactions, neuraminidase (in amounts employed in the tests) was treated similarly but in the absence of EDC.

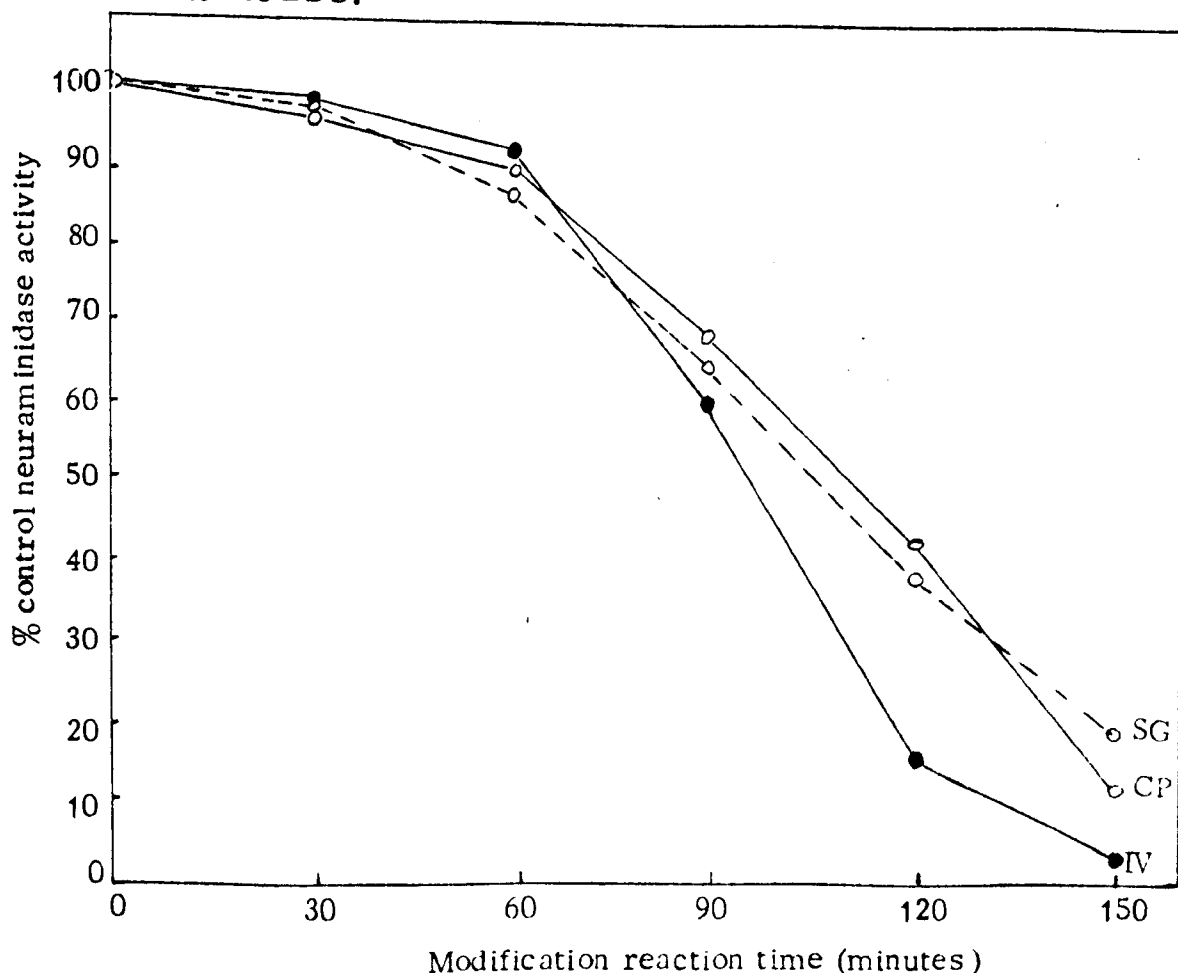


Fig. 3.14 Modification of neuraminidases with EDC. *S. griseus* (SG, 0.5 mg), *Cl. perfringens* (CP, 1.0 mg) and influenza virus (IV, 50 units).

(b) Protection by NANA or sialyllactose

To test the protection afforded by NANA in the modification of *S. griseus* neuraminidase with EDC as described above, the experiments were repeated in the presence of varying amounts of NANA (Figure 3.15). NANA was mixed with the enzyme and glycine ethyl ester before EDC was added. Similarly *Cl. perfringens* neuraminidase was modified in the presence of varying amounts of sialyllactose.

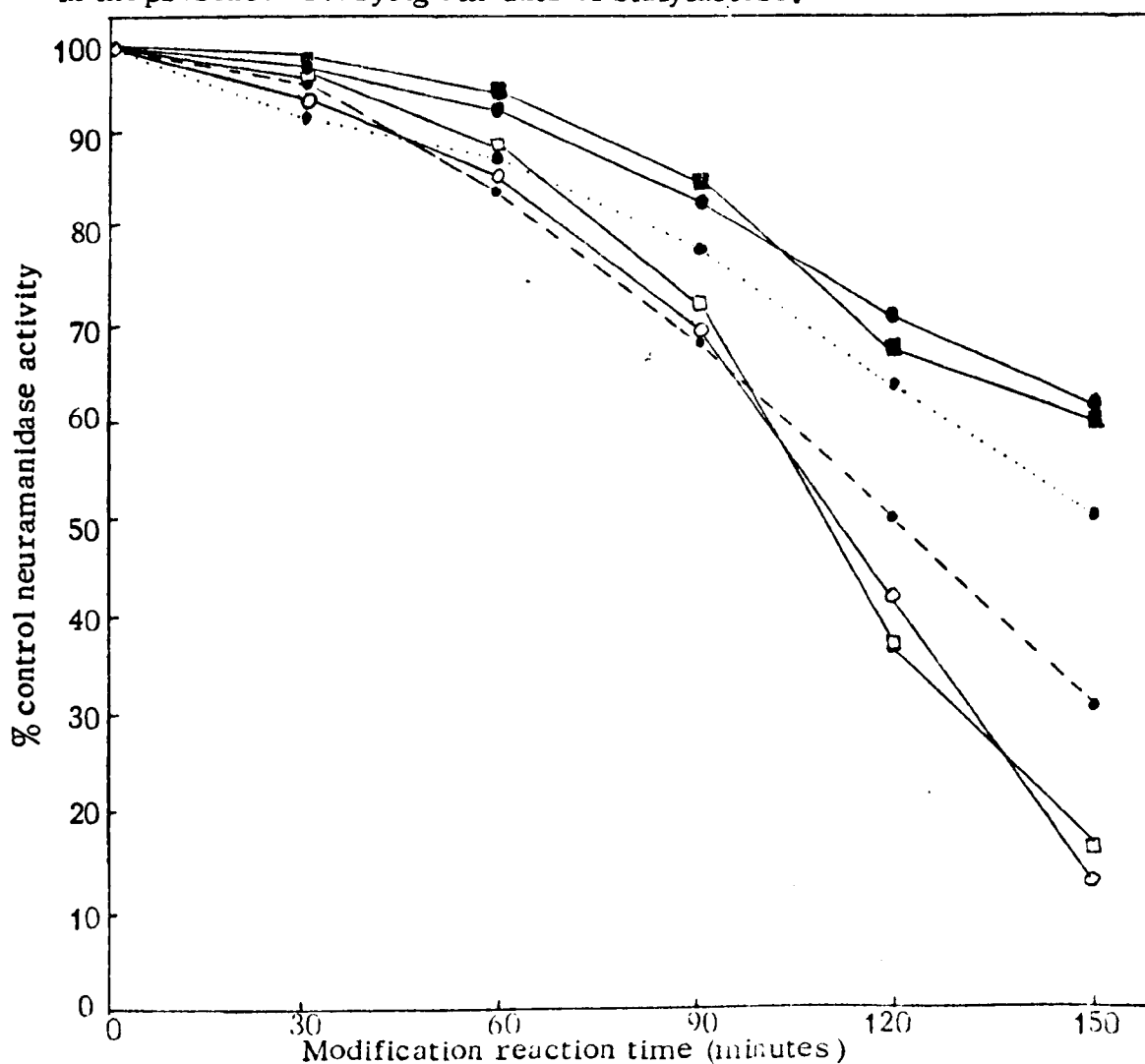


Fig. 3.15 Effect of EDC modification of *S. griseus* neuraminidase in the presence of: 10 mM (●—●), 30 mM (●—●) and 60 mM

(●—●) NANA. Control, in the absence of NANA, (○—○).

Modification of *Cl. perfringens* neuraminidase in the presence of 0.2 mg/ml (■—■) and absence (□—□) of sialyllactose.

(c) Incorporation of radioactively labelled glycine into *S. griseus* neuraminidase

(i) Synthesis of ^3H -glycine ethyl ester hydrochloride (^3H -GEE)

The method described by Blomquist et al. [270] was used to prepare radioactively labelled ^3H -GEE by refluxing thionyl chloride ethanol

and ^3H glycine (obtained commercially and diluted 10^5 -fold with unlabelled glycine). The product of the synthesis was recrystallised from absolute ethanol and compared with pure glycine ethyl ester of commercial origin by melting point determination, elemental analysis[†], NMR (D_2O) and paper chromatography.* The specific activity of the synthesised ^3H -GEE was determined. Table 3.2 summarises the analytical data of synthesised and commercially obtained glycine ethyl ester hydrochloride.

Table 3.2 Analysis of ^3H -glycine ethyl ester hydrochloride

Melting point $^{\circ}\text{C}$		Synthesised 146.3	Commercial 147.5
NMR (D_2O) signals		Exchangeable protons and ethyl moiety	Exchangeable protons and ethyl moiety
Paper chromatography		Major spot R_f 0.69 Minor spot R_f 0.38	One spot R_f 0.72
Elemental analysis [†]	C	34.45	(33.93)
	H	7.15	(7.14)
	N	10.4	(9.89)
	Cl	25.6	(25.04)
	Ash	0.78	-
Specific activity (cpm/nmole)		45	-

(II) Modification of *S. griseus* neuraminidase with ^3H -GEE

The procedure described in section 3.5.6(a) was repeated using 500 μg neuraminidase and ^3H -GEE instead of the unlabelled ester. The modification reaction was conducted in the absence of added NANA for 2 hours, at the end of which the modified enzyme (after gel filtration as described above) was precipitated with 10% trichloroacetic acid (TCA). The precipitate, after filtration on a cellulose membrane filter, was washed with 10% TCA, dried, and its radioactivity content was determined by standard liquid scintillation counting.

[†] Elemental analyses were performed by C.H.N. Analysis Ltd., South Wigston, Leicester. Values in brackets are calculated for $\text{C}_4\text{H}_{10}\text{ClNO}_2$.

* Paper chromatography was performed on Whatman No. 1 paper employing ethanol/water (1:1 by volume) and ninhydrin as developer.

Another sample of S. griseus neuraminidase (500 µg) was modified, in the presence of 50 mM NANA, using unlabelled glycine ethyl ester for 60 minutes, and the reaction contents were subjected to gel filtration of Sephadex G-25 as already described. The effluent protein fractions containing the modified enzyme were pooled and incubated with ^3H -GEE (1.3 M) and EDC for 60 minutes as before. The modified protein, after filtration as described above, was precipitated with 10% TCA, washed, dried, and its radioactivity content was determined (Table 3.3).

Table 3.3 Incorporation of ^3H -glycine in S. griseus neuraminidase

<u>Reaction</u>	<u>Amount enzyme modified (µg)</u>	<u>Incorporated ^3H-glycine (cpm)</u>	<u>Number of ^3H-glycine incorporated per 32000 M. Wt.*</u>
Total modification with ^3H -GEE (NANA absent)	500	46350	65.9
Unlabelled modification (NANA present) + ^3H -GEE	500	3485	4.9

3.5.7 Modification of tryptophan in S. griseus and Cl. perfringens neuraminidases

Modification of S. griseus and Cl. perfringens neuraminidases was carried out at room temperature using N-bromosuccinimide according to the method of Spande and Witkop [268]. Each enzyme in 0.1 M sodium acetate buffer, pH 4.0 (2 ml) was reacted with N-bromosuccinimide (NBS) in various molar enzyme:NBS ratios (Figure 3.16) in the presence or absence of 50 mM NANA or 0.2 mg/ml sialyllactose for 10 minutes before determining its specific activity.

* The number of carboxyl residues modified correspond to the number of moles ^3H -glycine incorporated, assuming the specific activity of the ^3H -GEE used to be 45 cpm/nmole, and a molecular weight of 32,000 for neuraminidase.

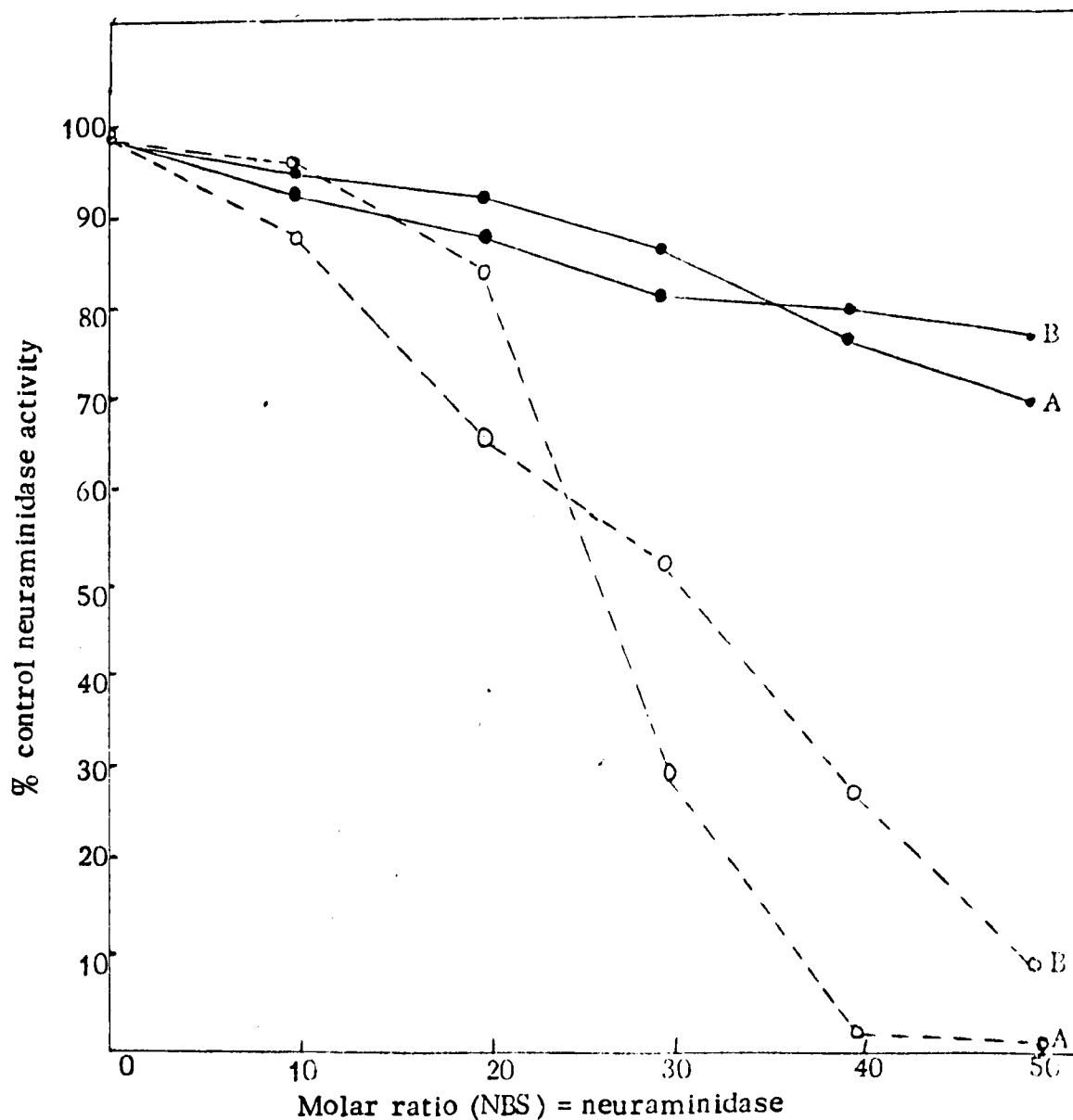


Fig. 3.16 The effect of NBS on *S. griseus* (A) and *Cl. perfringens* (B) neuraminidase activity in the presence (—●—●—) or absence (---○---) of 50 mM NANA or 0.1 mg/ml sialyllactose. As before, NANA was used to protect *S. griseus* neuraminidase while sialyllactose was used to protect *Cl. perfringens* neuraminidase.

In all these studies, the specific activities (enzyme activity/mg protein) were measured in order to afford a more accurate comparison than if just the neuraminidase activity was to be measured.

DISCUSSION

The quest for an understanding of the chemical basis of neuraminidase catalysis continues to represent a major segment of current biochemical research and some of the experimental avenues to this problem have been discussed above. In the present study the task has been the investigation of chemical modification approaches designed to gain understanding of the nature of the active site of neuraminidases. The correlation of loss of activity with modification of particular amino acid residues in *S. griseus*, *Cl. perfringens* and influenza virus neuraminidases is summarised in Table 3.4

Table 3.4

Effect of chemical modification on neuraminidase activity *

Modification procedure	Amino acids modified		% control activity after modification					
	Predominant	Side reaction	Without protection			In presence of NANA or SL		
			SG	CP	IV	SG	CP	IV
TNBS	Lys	Met, Cys	95	90	80	n.d.	n.d.	n.d.
Photooxidation	His	Met, Cys, Try, Tyr	85	70	50	85	70	60
Diethylpyro-carbonate	His	-	90	80	70	n.d.	n.d.	n.d.
2,4-pentanedione	Arg	Lys	5	15	20	n.d.	n.d.	n.d.
2,4-pentanedione + hydroxylamine	Arg	-	10	25	35	85	80	80
Phenylglyoxal	Arg	α -amino groups	5	15	n.d.	85	90	n.d.
EDC	Carboxyl groups (Glu, Asp)	Polymerisation Cys, Tyr	25	15	10	60	75	n.d.
NBS	Try	Tyr, Met, Cys, His, Arg, Peptide cleavage	0	5	n.d.	65	75	n.d.

* SL - sialyllactose.

n.d. - not determined.

SG - *S. griseus* neuraminidase.

CP - *Cl. perfringens* neuraminidase.

IV - Influenza virus neuraminidase.

The reaction of each of the three neuraminidases with the various modifying reagents achieved the inactivation of the enzyme to different extents. The most pronounced inactivation effect was obtained when the reagents known to modify arginine, tryptophan and carboxylic amino acids were reacted with the enzyme. In these modifications the resulting enzyme inactivation followed pseudo-first order kinetics and, in the case of arginine and tryptophan, modification was retarded by NANA or sialyllactose. These results are consistent with the likelihood that these amino acid residues are in or near the active site region of the enzyme and this proposition is further supported by the demonstration that the residues were protected by substrate or competitive inhibitor. The modification of carboxyl groups was not completely protected by NANA in the case of S. griseus neuraminidase or by sialyllactose in the case of Cl. perfringens neuraminidase, and therefore these amino acid groups probably participate much more in the catalytic than the substrate-binding events of the enzyme function. It is also possible that these groups may not be sufficiently protected on account of their weak binding with the inhibitor or with the substrate. Alternatively, they may be situated at positions near the active centre but where they do not maximally interact with the substrate or inhibitor. The modification of 4.9 carboxyl groups/mol S. griseus neuraminidase (Table 3.3, page 91) by EDC and glycine ethyl ester with simultaneous loss of 75% of enzyme activity is fairly specific since there are 65.9 free carboxyl groups in this enzyme as measured by the amount of ^3H -glycine ethyl ester. The value of 65.9 is close to that of 61.9 obtained from the sum of glutamic and aspartic acid residues as determined by amino acid analysis (Table 2.7, page 47).

In S. griseus neuraminidase, the incorporation of ^{14}C -phenylglyoxal showed that 1.3 out of 8.1 arginine residues per mol of the enzyme are protected by NANA and are possibly involved in the active site of the enzyme.

The observed reduced activities of the enzymes after reaction with TNBS or diethylpyrocarbonate or after photooxidation may be due to the operating side effects of these treatments. Viral neuraminidase, although

shown to lack free sulphydryl groups [211] and although its activity is known not to involve sulphydryl groups [252], has a tetrameric structure now known to depend on disulphide bonds [212]. Therefore treatments such as photooxidation known to affect cysteine, even if slowly, under the conditions employed, would be expected to show an effect on the enzyme activity. Although the effect of the modification procedures employed on the structure of neuraminidase was not investigated in these studies, it was possible to distinguish such effects from those occurring at or near the active site of the enzymes by performing the modifications in the presence or absence of a competitive inhibitor or substrate of the enzyme. Neuraminidase from Vibrio cholerae has been shown to lack sulphydryl and primary amino groups essential for catalytic activity [254, 272] although Groundwater in this laboratory [258] used TNBS to modify the V. cholerae enzyme and observed a 78% inactivation of its activity as a result of this treatment. However, this inactivation was not retarded by NANA. Parker *et al.* [188] were able to immobilise Cl. perfringens neuraminidase on cyanogen bromide-activated Sepharose and other supports, presumably through terminal amino groups, and although these amino groups were possibly not part of the active site, the treatment provides circumstantial evidence that the amino groups of this enzyme are not crucial for its activity. The results obtained in this study for this enzyme using TNBS are consistent with such suggestions. The slight reduction (10%) in the activity after the TNBS treatment of this enzyme may have been due to the side reactions of the reagent that affect sulphydryl groups which are probably crucial for the activity of the Cl. perfringens enzyme [43].

In the case of S. griseus neuraminidase, neither histidine nor lysine residues seem essential for the activity. This is in keeping with the results from the kinetic investigation of the effect of added metal ions on the enzyme (see page 54, Table 2.8). It is known that at increasing metal ion concentrations, primary amino groups, imidazole and carboxyl groups appear to co-ordinate with the metals [273]. Since the added metal ions did not affect the enzyme activity appreciably, these groups

were either not fully co-ordinated with the metal ions (especially the carboxyls which are quite numerous in this enzyme, see page 62 and Table 2.7) or altogether unnecessary for the activity of the enzyme. However, the evidence from the carboxyl modification of S. griseus neuraminidase would imply that the carboxyl groups are necessary for full enzymic activity.

Neuraminidases from different sources are immunologically distinguishable and are therefore an isodynamic group of enzymes which catalyse the same reaction but differ in a number of fundamental properties such as pH, ion requirement, substrate specificities and inhibition. Since these enzymes catalyse the same reaction, it is reasonable to propose that essentially similar catalytic events take place when the enzymes from different sources act. The activities of neuraminidases are necessarily difficult to compare because as well as possessing different structural properties, their substrates in themselves vary in structure depending on whether they are 2→3, 2→6 or 2→8 α-ketosides of sialic acid (see page 28, Fig. 2.1). Often the substrates used to assay and to characterise different neuraminidases contain a mixture of these ketosides whose absolute geometries must affect the interaction of neuraminidases possessing different structural environments to varying degrees. Drzeniek [274] carried out inhibitory experiments which demonstrated that the rate limiting factor of the enzymatic process is not the enzyme substrate-binding but the hydrolysis of the substrate. All sialic-acid-containing substrates could potentially bind to neuraminidase but differences can be expected with respect to the ease at which the neuraminidase can elute from different substrates by splitting off sialic acid. The elution from different substrates of different neuraminidases may thus depend on the intimate interaction of the substrate with the enzyme. This will in turn be influenced by various factors relating to the structure of the active site of the enzyme, for example the molecular environment of the active site, the size of the active site, etc. The results of the modification studies in the present study indicate that the three types of neuraminidases have common amino acid residues

essential for their activity, namely arginine, tryptophan and carboxylic amino acids. The results of previous modification studies on neuraminidases by other workers were mentioned above (see pages 70-72) and although a few are contradictory to the result of the present study, it is interesting to consider the aspects in which the participation of arginine, tryptophan and carboxyls would fit in with the activity of neuraminidase or related enzymes.

Neuraminidase catalyses the hydrolytic cleavage of the α -ketosidic linkage that joins sialic acid to glycoproteins, glycolipids and oligosaccharides. The presence of a free terminal α -carboxyl group of the substrate is a strict specificity requirement of this enzyme [247, 248] and because of such specificity for the anionic form of the sialyl substrate, it is likely that a positively charged recognition site at the active centre of the enzyme would be required for the binding of the substrate. The involvement of a metal ion for this purpose has not been definitively implicated in the catalytic or binding function of neuraminidase. A positively charged amino acid residue such as lysine, histidine or arginine, which exist in their ionised form over the usual pH range of enzyme stability could be considered as the most likely alternative. The experiments reported above have excluded participation of lysine or histidine residues in binding functions. In contrast the results available from these experiments indicate that arginine is critical for enzyme activity. At least 1000 of the more than 1500 enzymes now known act on negatively charged substrates or require anionic cofactors, and a number of reports have suggested that arginyl residues often serve as the complementary, positively charged recognition sites.

Tryptophan, which was also found to be essential for neuraminidase activity in the present study, has been shown to take active part in carbohydrate-protein interactions in some carbohydrate-binding proteins, for instance lysozyme [275], some lectins [276] and α -mannosidase from *Phaseolus vulgaris* [277], to mention a few. Further, the neuraminidases from an *Arthrobacter* species [210], *Cl. perfringens*, and influenza virus [253] were all shown to possess an essential tryptophan residue either in the active site or in maintaining the intact structure of the protein molecule necessary for the activity of neuraminidase.

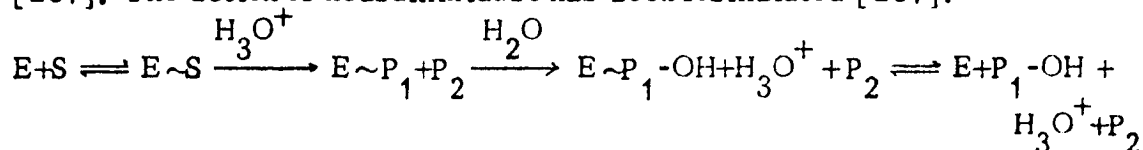
The finding in the present study that carboxyl groups appear to be involved in the catalytic activity of neuraminidase is analogous to the findings for some other glycosidases such as sucrose-isomaltase [278], lysozyme [275] and *Aspergillus wentii* β -glucosidase [279]. The participation of a dissociable group such as a carboxyl group, active in enzyme action in the protonated form, with a pH dependence similar to that of the carbodiimide reaction may be suggested by the optimal pH (about 5.0) of several neuraminidases, and is the most likely candidate as a proton-donating amino acid group. In the case of lysozyme [275] it has been suggested that the most reasonable explanation of the catalytic activity is that two carboxyl groups (Asp 52 and Glu 35) which are close to the inhibitor-binding site function act as base and acid to effect hydrolysis of susceptible glycosidic bonds. In the case of neuraminidase only the report of Homquist [242] dealing with the effect of carboxyl modification of *Vibrio cholerae* neuraminidase with ethyl diazoacetate has appeared. However, the fact that α -ketosides of sialic acid bearing an anionic group in the polyhydroxy side chain are neither substrates nor inhibitors of the enzyme [240] is suggestive of the possibility that the active site of neuraminidase may be associated with a negatively charged residue such as a carboxyl group.

Speculations on the mechanism of neuraminidase action

The mechanism of nonenzymatic hydrolysis of glycosidic and ketosidic bonds is now established [280] as a unimolecular reaction involving fission of the carbonyl-carbon-oxygen bond with a cyclic carbonium or oxocarbenium ion as an intermediate. Cordes and Bull [280] have suggested the following as the possible mechanisms for the cleavage of carbonyl-carbon-oxygen bonds:

- (a) Protonation of the oxygen atom followed by the rate determining formation of an oxocarbenium ion (specific acid catalysis).
- (b) Concerted proton transfer and cleavage of the carbonyl-carbon-oxygen bond with formation of an oxocarbenium ion (general acid catalysis).
- (c) Attack by nucleophiles.

It is thought that a similar mechanistic pathway involving an intermediate oxocarbenium ion, possibly formed following protonation of a glycosidic oxygen atom by an acid group, e.g. protonated carboxylic side chain, could function in the enzyme-catalysed hydrolysis of glycosidic bonds. Indeed, such an intermediate carbonium ion is known to be formed in the case of lysozyme [275] and *Vibrio cholerae* neuraminidase [281]. The action of neuraminidase has been formulated [281]:



In this sequence a cyclic oxocarbenium ion of N-acetylneuraminic acid (Fig. 3.17A) is assumed to react with water to produce the β -anomer directly or by anomerisation of an initially formed α -anomer.

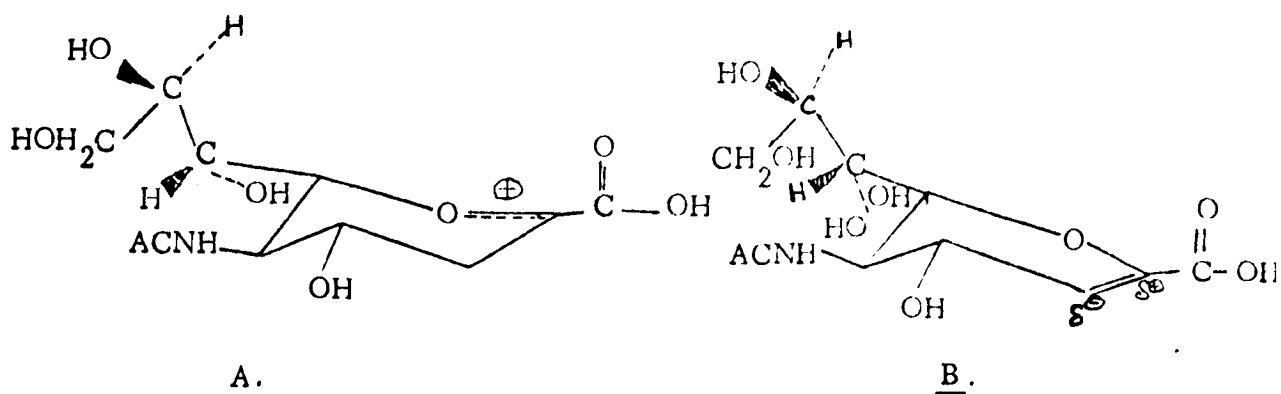


Figure 3.17 A. Structure of the cyclic oxocarbenium ion of N-acetylneuraminic acid.

B. 2-deoxy-2,3-dehydro-N-acetylneuraminic acid

In the reaction sequence depicted above, P_1 represents the cyclic oxocarbenium ion, $P_1 - OH$, the free N-acetylneuraminic acid, P_2 the released aglycone, E the enzyme and S the substrate. Flashner et al. [282] have found that 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (Figure 3.17B) and its methyl ester are transition state analogues for neuraminidase and their structural resemblances to the oxocarbenium ion support the hypothesis that an oxocarbenium-like ion is generated during substrate catalysis of neuraminidase.

Since the aglycone moiety of natural or synthetic substrates for neuraminidase pose little steric hindrance to the enzyme, it is most probable that the enzyme approaches its substrate from the sialic acid moiety, and assuming the neuraminidase possesses a cleft or pocket into which the sialic acid moiety fits (Figure 3.18), it is reasonable to suppose that a hydrophobic amino acid residue such as tryptophan would interact with the polyhydroxy chain.

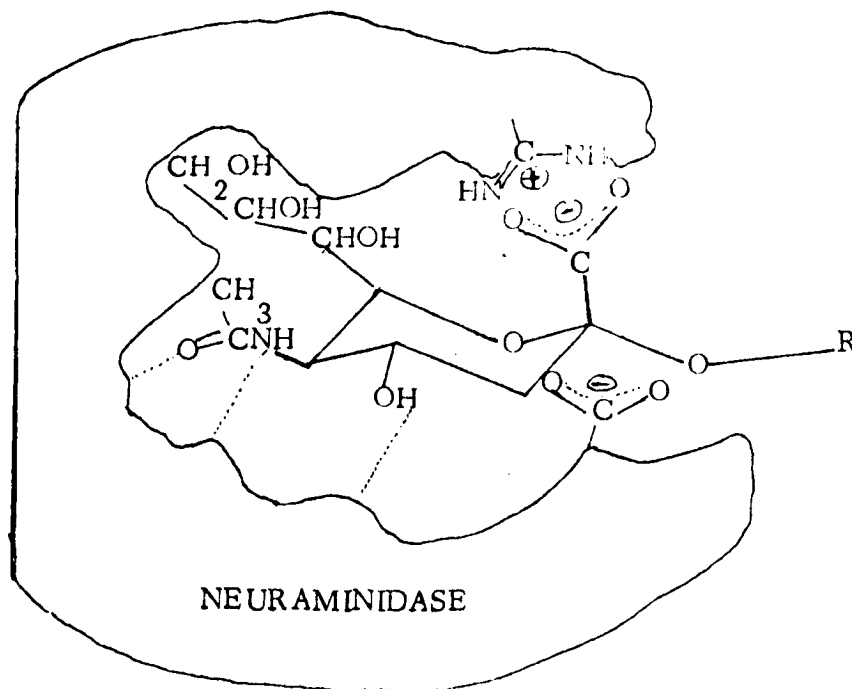


Figure 3.18 Possible mechanism of substrate binding by neuraminidase

The free carboxyl of the substrate, which is obligatory for the action of neuraminidase, may function by binding to a positively charged residue such as arginine and may in the process distort the sialic acid molecule from its normal 2C_5 conformation into that approaching a half-chair conformation [282]. Further, the guanidino groups of arginine may have a function beyond that of merely binding the substrate, such as accelerating the hydrolysis reaction by neutralising the negative charge on the substrate carbonyl-carbon-oxygen and partially polarising it, an effect which would tend to electrostatically stabilise the cyclic oxocarbenium ion formed by the cleavage of the glycosidic bond.

Involvement of one or more carboxyl groups in the catalytic mechanism of neuraminidase is a possible working hypothesis by analogy with the well-known example of lysozyme [275] in which a carboxyl group promotes the formation of an intermediate carbonium ion by donating a proton to the glycosidic oxygen. Thus, in the case of neuraminidase, the following Figure (3.19) may be the mechanism by which α -ketosides of sialic acid are cleaved.

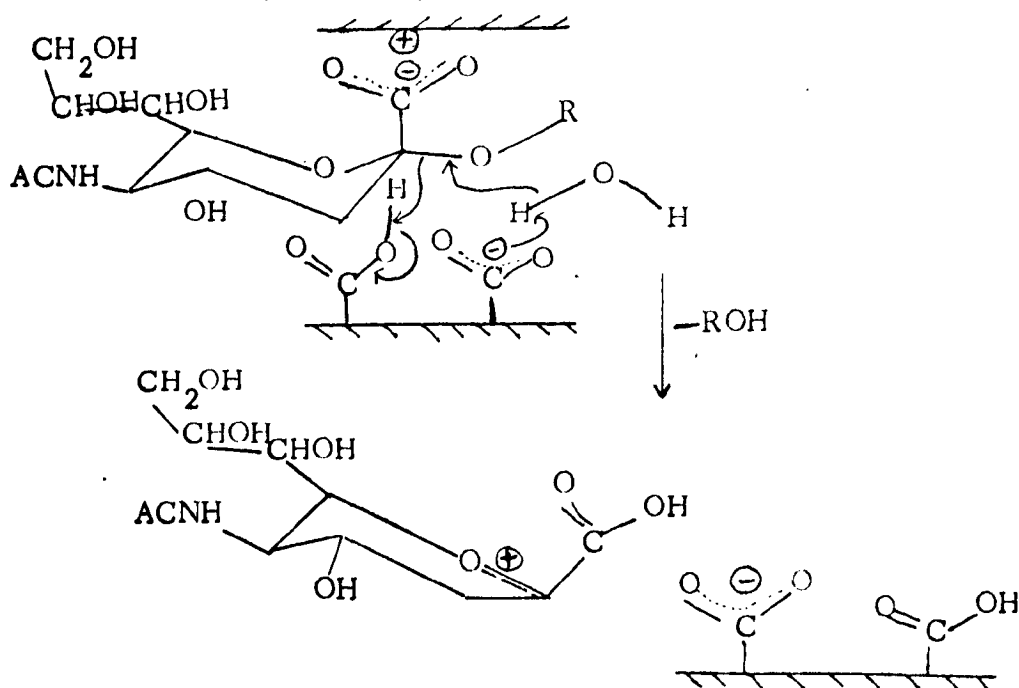


Fig. 3.19 Possible hydrolytic mechanism of neuraminidase

The release of the free sialic acid after hydrolysis would probably be facilitated by an electrostatic repulsion between the formed positively charged oxocarbonium ion and the positively charged guanidino group of arginine. In addition to this, the release of the free sialic acid (breakdown of $E \sim P_1 - OH$) is effected by the rapid anomerisation of sialic acid to its β -anomer or by the direct formation of the latter [242, 281].

3.7 Conclusion

The importance of neuraminidase has already been discussed above. The results from the present studies on neuraminidases can at the moment afford only a hypothetical explanation of the mechanism of neuraminidase action. More light can be expected from other approaches such as X-ray studies, transition-state affinity label analysis, peptide mapping techniques, etc. With the improved methods now available for the purification of neuraminidase and the increasing task force of enzymologists and protein chemists now interested in the enzyme, it may not be long before the knowledge on the mechanism of neuraminidase is complete.

APPENDIX I

Practical application of purified *S. griseus* neuraminidase

Treatment of Interferon with neuraminidase

Most interferons are probably glycoproteins. While investigating the functional and structural composition of the carbohydrate moiety, Dorner et al. [283] treated rabbit interferon with neuraminidase as a result of which they observed considerable decrease in its charge heterogeneity on isoelectric focussing. However, the neuraminidase utilised in these studies had been purified by an affinity chromatographic method [174] which has now been shown to be non-specific [206, 207]. Further, the separation procedures employed in isoelectric focussing were assumed to achieve total dissociation of the protein complexes involved in the study.

In the studies reported below, the extensively purified neuraminidase from *S. griseus* neuraminidase was used to treat human fibroblast, leucocyte and lymphoblastoid interferons, after which the isoelectric focussing method of O'Farrell [284], in which full dissociation of protein complexes is known to occur, was used to analyse the enzyme-treated interferons.

As well as testing the validity of studies by Dorner et al. [283], the present studies also aimed at investigating any sialylation differences in the three interferons tested.

*Leucocyte interferon (2500 units), lymphoblastoid interferon (2500 units) and fibroblast interferon (1000 units) were each incubated with purified *S. griseus* neuraminidase (50 µg) and 0.05 M sodium acetate buffer, pH 5.3 (250 µl), at 37° C for 3 hours. The corresponding controls were similarly incubated in the absence of neuraminidase. After the incubations, the samples were analysed by isoelectric focussing essentially as described by O'Farrell [284] except that 2-mercapto-ethanol was omitted from all buffers and 0.25% SDS was included in the sample buffer. After isoelectric focussing, the gels were either

* The three interferons were provided and assayed by Dr. J.M. Morser.

stained for protein with Coomassie blue or sliced into 0.25 cm segments, each of which was placed in phosphate buffered saline (PBS) and dialysed exhaustively against PBS before its interferon content was determined. The pH gradient in the isoelectric focussing gels was determined and the isoelectric points of the three interferons were estimated (Figure A.1).

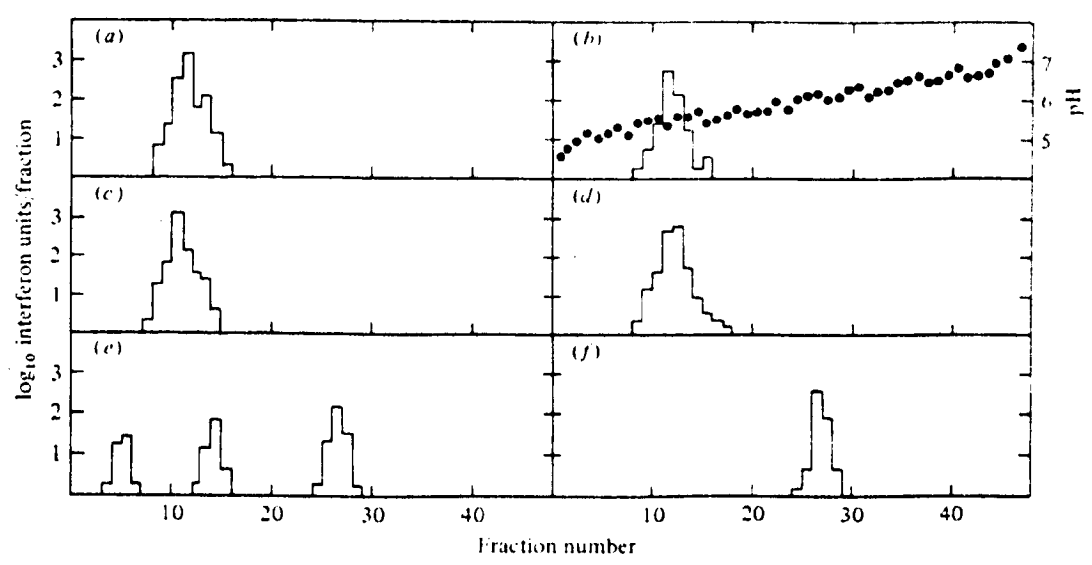


Figure A.1 Isoelectric focussing of human interferons; (a) leucocyte interferon, (b) leucocyte interferon treated with neuraminidase, (c) lymphoblastoid interferon, (d) lymphoblastoid interferon treated with neuraminidase, (e) fibroblast interferon, (f) fibroblast interferon treated with neuraminidase. pH.

Fetuin (150 μ g) was incubated with neuraminidase (50 μ g) at 37 $^{\circ}$ C for 0, 10, 30, 40 and 60 minutes, after which incubation the samples were analysed by isoelectric focussing as described for interferon above, and stained for protein (Figure A.2).

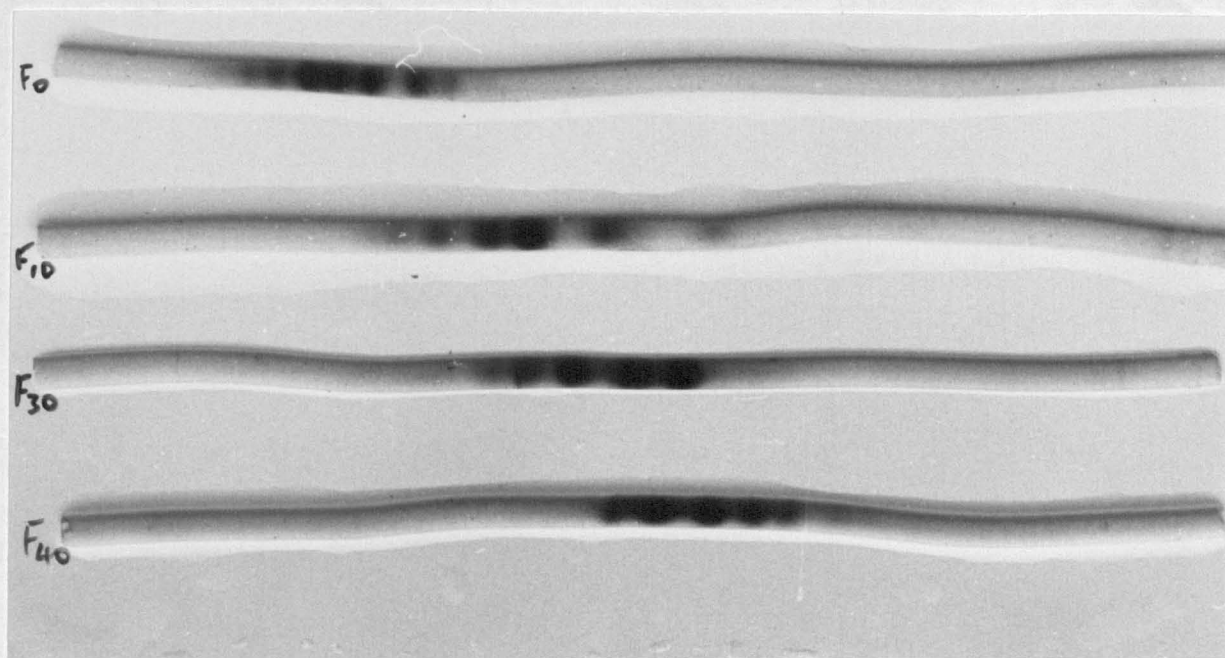


Figure A.2 Isoelectric focussing of neuraminidase-treated fetuin. F_0 - F_{40} fetuin treated with neuraminidase for the times (minutes) indicated by subscript.

Fetuin treated with neuraminidase for 60 minutes or longer (not shown in Figure A.2) was identical to F_{40} on isoelectric focussing. The amount of sialic acid produced in each incubation was determined and compared with the corresponding electrophoretic migration. The fetuin used in these studies (prepared by the method of Spiro [214]) appeared as multiple bands on isoelectric focussing in contrast to that prepared by the same method and reported to be homogeneous [214]. Therefore, to measure the migration of the bands, the midpoint of the band area was chosen and its distance from the origin was measured.

From Figure A.1 it would seem that only human fibroblast interferon was significantly altered by treatment with neuraminidase. Prior to the enzyme treatment, three peaks of fibroblast interferon activity could be detected with isoelectric points 5.2, 5.8 and 6.3. After treatment with neuraminidase, only one peak of interferon activity with an isoelectric point of 6.3 was detected. In the case of lymphoblastoid and leucocyte interferons, no change in their isoelectric points (5.5 and 5.3 respectively) following their treatment with neuraminidase could

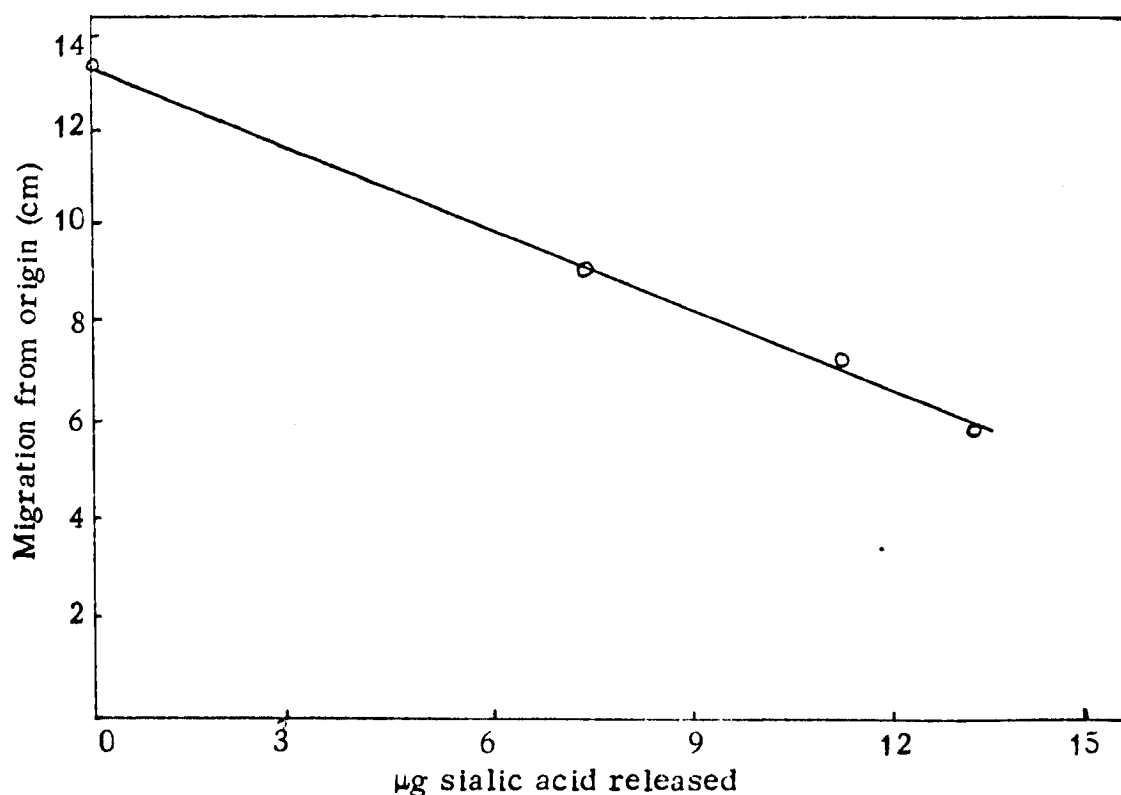


Figure A.3 Relationship between the sialic acid content of fetuin and its corresponding migration in isoelectric focusing.

be detected. In a related study, Mogenson *et al.* [284] also observed that the isoelectric focussing pattern of human leucocyte interferon was not changed appreciably by treatment of the interferon with neuraminidase.

The isoelectric focussing pattern of fetuin (Figure A.2) was changed appreciably by treatment of this glycoprotein with neuraminidase. The three forms of fibroblast interferon, separable on the basis of their intrinsic charge would reflect on their sialic acid content being more acidic the more sialic acid they contain. The form of fibroblast interferon with an isoelectric point of pH 6.3, both leucocyte and lymphoblastoid interferons as well as fetuin treated with the enzyme for 40 minutes or longer, probably lack neuraminidase-susceptible sialic acid residues.

It is interesting that two species of fetuin differing by as little as 0.1 µg sialic acid content may be 'visualised' as two bands at least 0.5 cm apart (Figure A.3). The isoelectric point of untreated fetuin was found to be about 3.5; after treatment with neuraminidase the iso-

electric point was raised to pH 5.4. This analytical technique has great potential in assessing the content of sialic acid in substances such as interferon which are normally available in amounts too low to afford quantitative estimation of sialic acid. The technique may also be applied to indirectly assess neuraminidase amounts since there is a direct relationship (Figure A.3) between the sialic acid content and isoelectric point.

APPENDIX II

A.2 Attempt to Design a Radioimmuno Assay for Sialic Acid

A.2.1 Introduction

Most of the problems associated with the determination of free sialic acid (and neuraminidase activity), some of which have been mentioned in Chapter 2, relate to the limitations in the specificity and/or sensitivity of the techniques employed. The rapidly growing list of new methods used to quantitate sialic acid is suggestive of the dissatisfaction expressed by various investigators at the currently available techniques.

The techniques of radioimmuno assay, first developed for the measurement of hormones, has expanded over the last two decades to include the detection of many other biological substances. The reasons for such wide application of this technique derive from its high sensitivity, specificity and precision compared to other analytical methods. In a typical radioimmunoassay, the antibodies (Ab) to the biological substance of interest (antigen, Ag) are prepared and used to determine the concentration of the antigen, following observations in which the radiolabelled antigen molecules compete with the non-labelled antigen molecules for a limited number of binding sites on the antibodies. Figure B.1 summarises the reactions involved in radioimmunoassays [286].

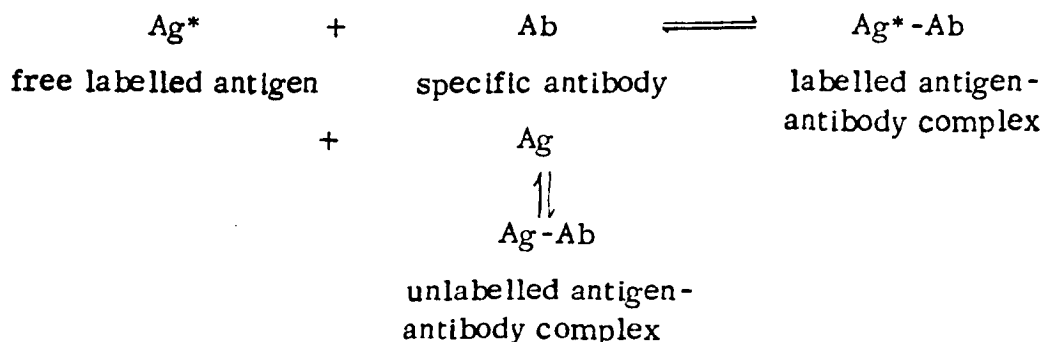


Figure B.1 Summary of the principles of a radioimmunoassay

The mixture of Ab, Ag and Ag*, in which there is a fixed amount of Ab but a relative excess of Ag, is incubated to allow establishment of an equilibrium before the antigen-antibody complexes (Ag-Ab and Ag*-Ab)

are separated from free unbound Ag^* and Ag . The radioactivity of complexed or free Ag^* or both is measured. When increasing amounts of unlabelled antigen are added to the assay, the limited binding sites of the antibody can bind less of the radiolabelled antigen. A diminished binding of labelled antigen offers evidence for the presence unlabelled antigen. The concentration of the unlabelled sample antigen can then be obtained by comparing the observed inhibition of labelled antigen binding with that obtained by standard solutions containing known amounts of antigen.

Any compound can be measured by the radioimmunoassay technique provided that:

- (a) it can be made immunogenic;
- (b) it is available in pure form;
- (c) it can be radioactively labelled;
- (d) there is a technique available to separate the antibody, complexed from the free compound.

Both unlabelled and labelled N-acetyl neuraminic acid are available commercially, but neither antibodies to sialic acid nor attempts to design a radiolimmuno assay for its determination have been reported.

A number of reports (especially on tumour immunology) have shown that sialic acids may play a peculiar negative role in masking antigenic determinants expressed by tumour cell surfaces [287] or normal cells [288] or soluble sialoglycoproteins [53]. Although this would reflect on the poor immunogenicity of sialic acid, there are several reports in which sialic acid has been shown to be an immunodeterminant sugar.

It has long been known that blood group specificities are determined by carbohydrate moieties of erythrocyte glycoproteins and glycolipids. Sialic acids, in particular, are responsible for the antigenic specificities associated with the MN [289], F [290], Pr_1 and Pr_2 [291] systems. While investigating the in vitro relationships of sialic acids to the A, B, M, N, C, D and E agglutinogens of the human erythrocytes

by means of the Landsteiner haptens inhibition test, Rule [292] obtained results which suggested that sialic acid is a major constituent of the D(Rho) and MN agglutinogens and showed that both colominic acid (from E. coli K 235 L⁺C1) and N-acetylneuraminic acid bind to anti-D. In other studies, Lisowka and Roelcke [293] showed that after periodate oxidation of red cell glycoproteins resulting in sialic acids with shortened polyhydroxy side chain, the Pr₁ and MN antigens were inactivated. Prat and Comoglio [294] obtained antisera solubilised membrane antigens from a mouse plasma cell tumour and by radioimmunoprecipitation techniques showed that the removal of sialic acid residues, with Vibrio cholerae neuraminidase, from the antigens significantly lowered the precipitation curves; in a competitive radioimmunoassay the desialysed antigens only partially inhibited the binding of radiolabelled antibodies to native antigens. Serological investigation of a mucopolysaccharide, obtained from the S-form of Salmonella ngozi, built up predominantly of sialic acid and hexosamines yielded results, the interpretation of which identified N-acetylneuraminic acid as the immunodeterminant sugar [295]. A polysaccharide isolated from Neisseria meningitidis group C and identified as a homopolymer of sialic acid has been shown to be immunogenic in rabbits and sheep [296] and in a recent investigation of the type III polysaccharide isolated from group B Streptococcus, sialic acid was identified as the major chemical constituent of the antigen, accounting for more than 25% of its carbohydrate [297]. Egan et al. [298] isolated a polysaccharide antigen from E. coli strain Bos-12 which was characterised as a polymer of sialic acids in 2'→8 linkages similar to colominic acid. Sialic acid has also recently been shown to be involved in the antigenic determinant of a glycopeptide from fetuin [299], Figure B.2, in contrast to an earlier report [300] in which fetuin was judged to be devoid of any carbohydrate antigenic determinants.

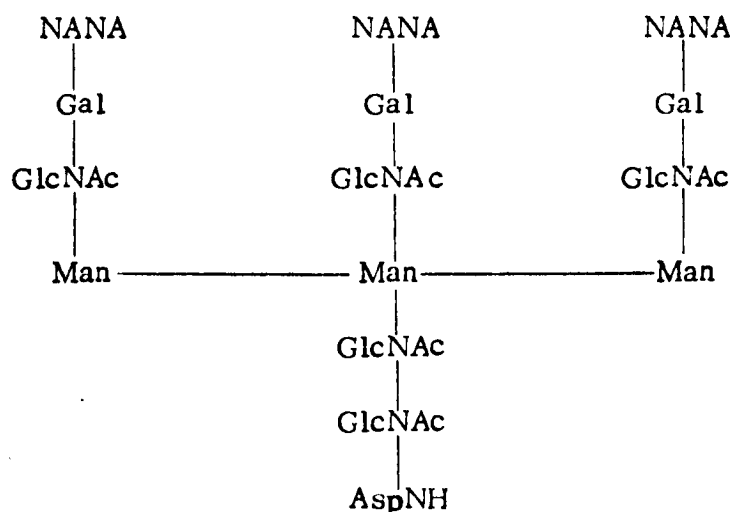


Figure B.2 Structure of possible carbohydrate immunodeterminant moiety in fetuin [299]. NANA is N-acetylneuraminic acid.

In the present study, colominic acid and fetuin were used as antigens against which antibodies were raised in rabbits; attempts were made to isolate antibodies specific to sialic acid with which to design a competition assay for the determination of sialic acid concentrations.

A.2.2 Experimental Procedure

New Zealand white rabbits were immunised at intervals of two weeks, each time with fetuin (20 mg/ml in PBS) or colominic acid (15 mg/ml in PBS) emulsified 1:1 in Freund's complete adjuvant and injected subcutaneously in the neck. Control bleeds were taken from the animals before immunisation. Test bleeds were taken from the animals six days after the second and subsequent injections of fetuin or colominic acid and the immunisation programme was continued until there was no further increase in the antibody titre. The blood collected separately from the rabbits injected with fetuin and from those injected with colominic acid was allowed to clot for 2 hours at room temperature and the sera were decanted from the clots and centrifuged at 4°C at 1500 x g for 30 minutes. The serum thus collected was subjected to various immunological tests [301] for antibody activity.

(a) Interfacial ring test

Serum (0.1 ml) from fetuin- or colominic acid-injected rabbits was placed in a small Durham tube and 0.1 ml of fetuin (15 mg/ml in PBS) or colominic acid (15 mg/ml in PBS) respectively was carefully added. After the second injection, a precipitation ring was observed in the tubes to which antiserum to fetuin and fetuin were present; no similar ring was observed in the case of colominic acid even after the seventh injection of colominic acid into the rabbits. Pure sialic acid also did not form a precipitation ring when added to either antifetuin or anticolominic acid sera.

(b) Immunodiffusion

The double diffusion technique was conducted on microscope slides coated with 2% agar (Noble, special for electrophoresis) gels in 0.08 M barbitone buffer, pH 8.2. Precipitation lines were visible in the case of fetuin antiserum (Figure B.3) but not collected from colominic acid-injected rabbits. The gels were dried and stained with Coomassie blue.

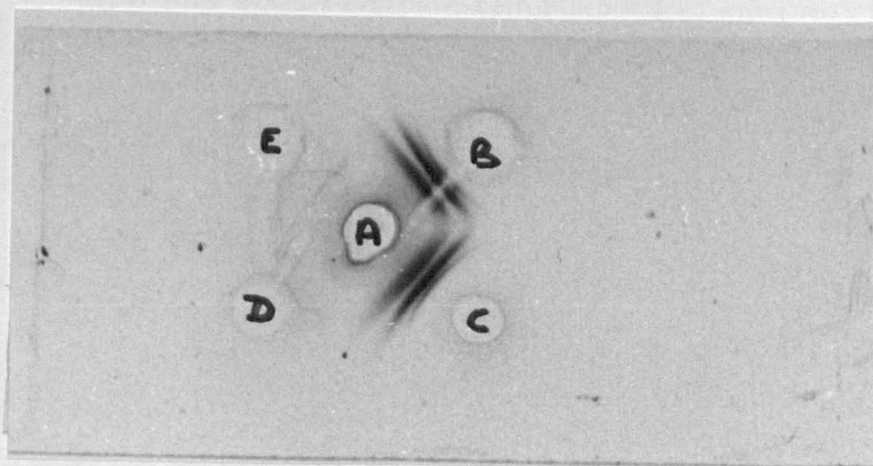


Figure B.3 Double immunodiffusion of rabbit antifetuin antiserum.

A, diluted 1:1 with PBS . B fetuin (5 mg/ml). C, desialysed fetuin*. D, N-acetylneuraminic acid (5 mg/ml).
E, colominic acid (5 mg/ml).

* Desialysed fetuin was prepared by incubating fetuin (1 ml, 5 mg/ml in 0.05 M sodium acetate buffer, pH 5) with neuraminidase (0.5 ml, 0.5 mg/ml in 0.05 M sodium acetate buffer, pH 5) from Clostridium perfringens at 37⁰ C for 6 hours. The incubation mixture was then exhaustively dialysed against PBS before being used in the immunodiffusion test. Further treatment of this desialysed fetuin with neuraminidase did not release any sialic acid as measured by the thiobarbituric acid test [196].

(c) Farr assay

The Farr assay involves the reaction of a radiolabelled antigen with antibody followed by precipitation of the resulting antibody-antigen complex with saturated ammonium sulphate. Since ammonium sulphate added to serum to 50% saturation precipitates most immunoglobins, leaving other serum proteins in solution, the radioactivity associated with the precipitate formed from a mixture of antibody and antigen will be due to the labelled antigen complexed with antibody.

It became interesting to find out if there were antibodies to sialic acid with which they formed soluble complexes or to which they bind by primary interactions - possibilities that could not be detected by immunological techniques which depend on secondary interactions and formation of insoluble antibody-antigen complexes.

¹⁴C-labelled N-acetylneuraminic acid* (20 μ l) was added to each of the centrifuge tubes containing varying dilutions of antiserum (to fetuin or columnic acid) or control serum. The contents of each tube were mixed and incubated at 4⁰ C for 3 hours. PBS was added to each tube to bring the final volume to 0.6 ml and after mixing, saturated ammonium sulphate (0.6 ml) was added; the contents of each tube were thoroughly mixed and allowed to stand for 20 minutes before centrifuging at 3000 x g for 15 minutes. The contents of each tube were completely transferred

* N-acetyl[4, 5, 6, 7, 8, 9-¹⁴C] neuraminic acid commercially obtained (259 mCi/mmol) was diluted about 100-fold with the unlabelled N-acetylneuraminic acid. The working solution had a specific activity of 2150 cpm/ μ l.

and filtered over a fibreglass filter. The precipitates on the filters were washed with 50% saturated ammonium sulphate solution (5.0 ml) and the filters were dried and their radioactivity content was determined by scintillation spectrometry (using Triton X-100 butyl PBD scintillant).

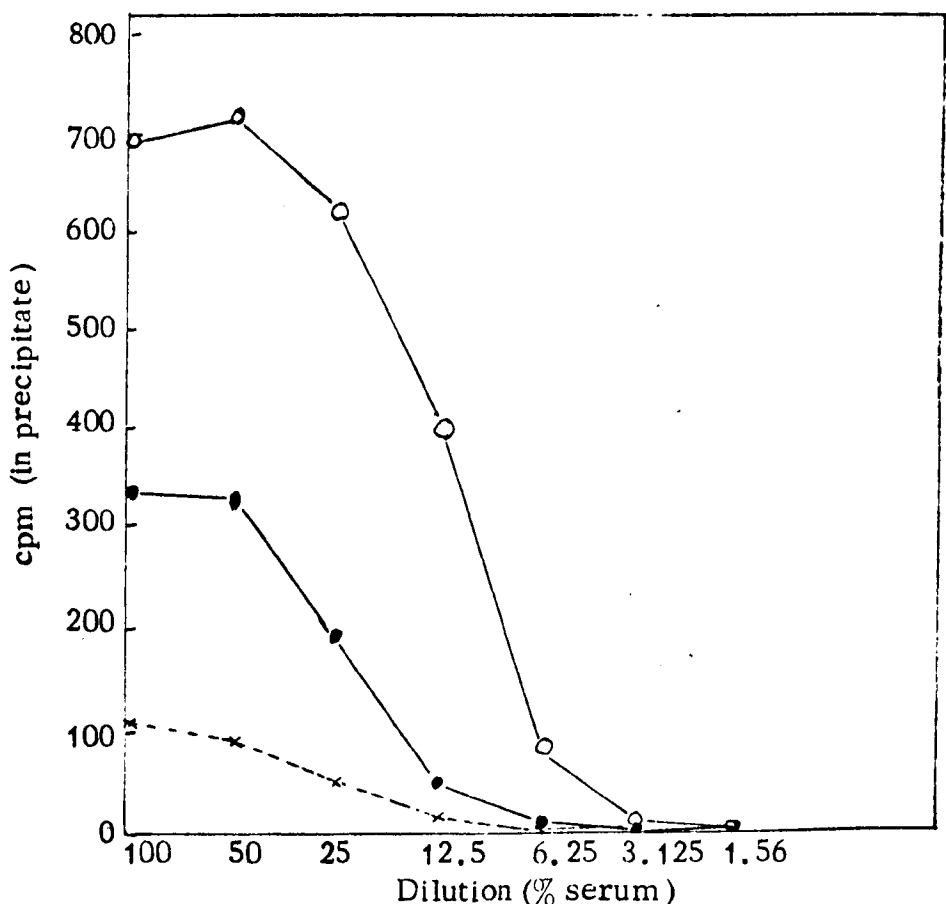


Figure B.4 [^{14}C] NANA binding capacity of antiserum to colominic acid (—○—○—) and fetuin (—●—●—) determined by the Farr assay. Control serum (---x---x---).

(d) Equilibrium dialysis

A microequilibrium dialysis apparatus similar to the one described by Neuhoﬀ Kiehl [302] (Figure B.5) was used. This apparatus consisted of a number of pairs of small chambers, each capable of holding a volume of 0.8 ml. The chambers in each pair were separated by a dialysis membrane.

Antibody immunoglobulins were prepared from the sera by precipitation with ammonium sulphate. Saturated ammonium sulphate

(100% in distilled water) was added to a final concentration of 45% (v/v) to serum diluted 1:1 with PBS. The mixture was stirred at room temperature for 30 minutes and centrifuged ($2000 \times g$) for 15 min at 4°C . The precipitate was washed with 45% saturated ammonium sulphate three times by centrifugation. The precipitate was dissolved in a minimum volume of PBS and exhaustively dialysed against PBS at 4°C . The protein concentration of the resulting solution was determined by its absorbance at 280 nm, assuming that an O.D. value of 1.0 (1 cm cuvette) is equivalent to a gamma globulin concentration of 0.70 mg/ml [301]. Using this method, an average of 160 mg protein was obtained from 25 ml of serum.

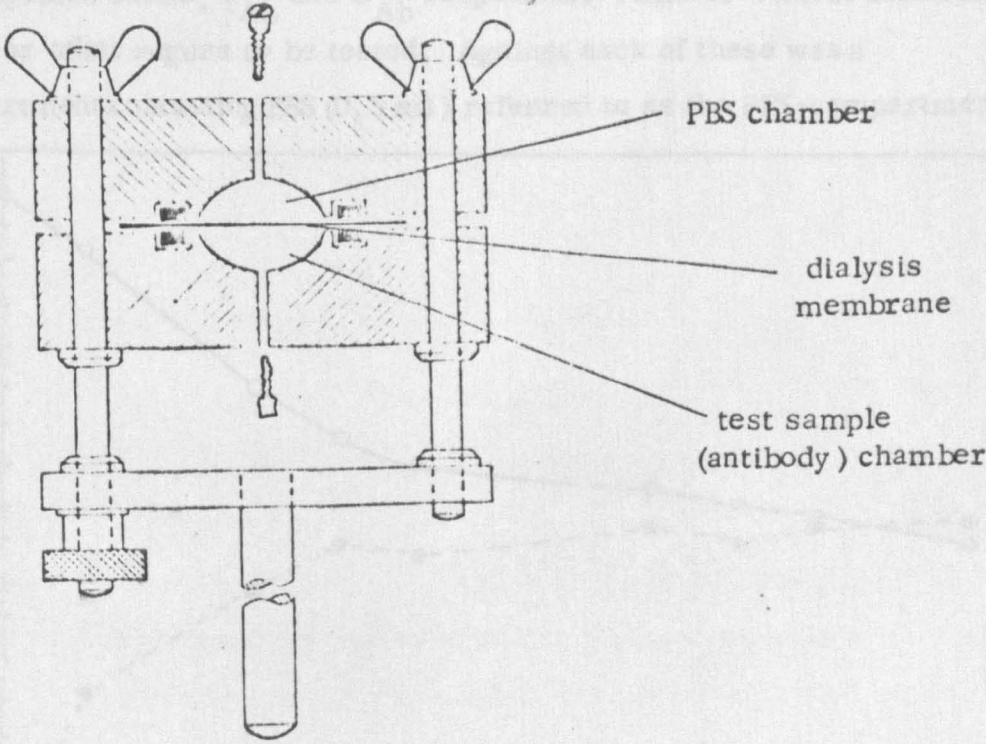


Figure B.5 Diagram of a microequilibrium dialysis apparatus

A binding curve was obtained by studying the equilibration of constant amounts of antibody with various concentrations of ^{14}C -NANA on opposite sides of a dialysis membrane. For a single point of a binding curve, 3 compartments were set up.

(i) The buffer control which was used to determine whether the equilibrium was established (Figure B.6) and also the amount of ^{14}C -NANA adsorbed to the dialysis membrane and to the apparatus.

(ii) An immunoglobulin fraction obtained from control serum which served to determine the amount of ^{14}C -NANA non-specifically bound to immunoglobulins.

(iii) A compartment containing the antibody solution to be tested (the immunoglobulin fraction prepared from the sera of fetuin- or colominic-acid-injected rabbit, F_{Ab} and C_{Ab} respectively) with or without unlabelled NANA or other sugars to be tested. Against each of these was a compartment containing PBS (0.5 ml) referred to as the PBS-compartment.

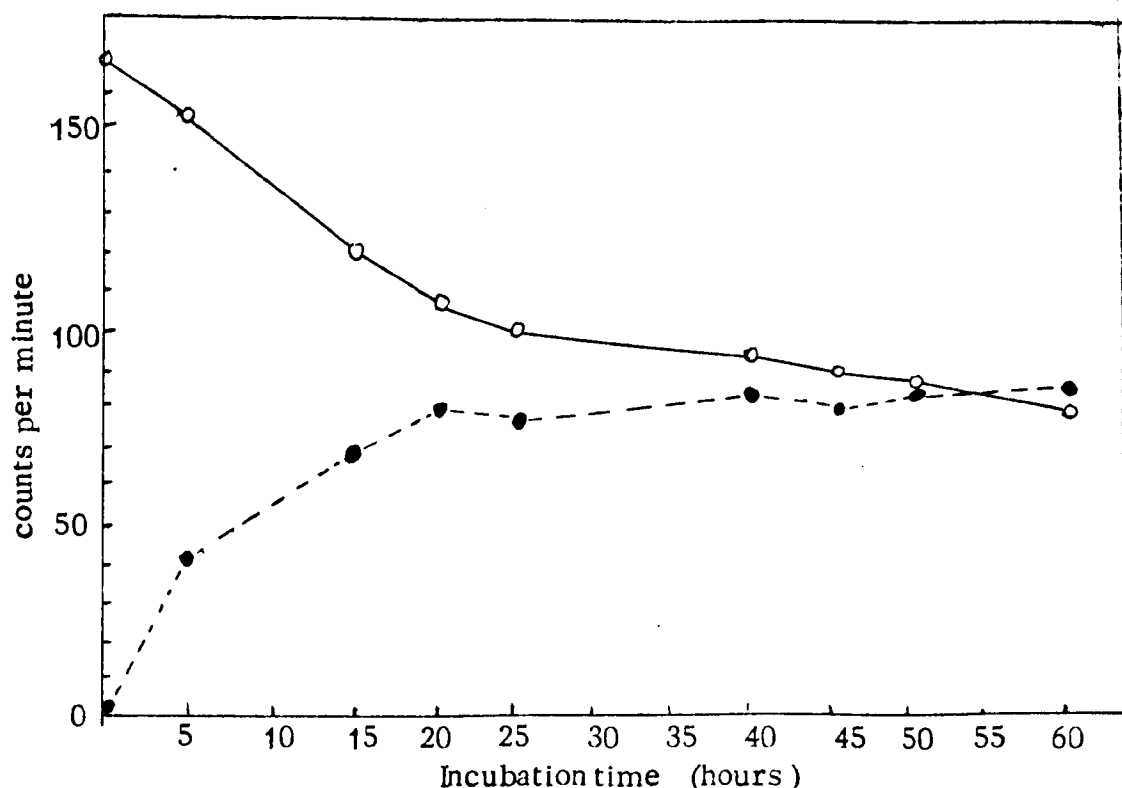


Figure B.6 Determination of completion of equilibrium. At the start of this experiment, one compartment contained PBS (0.5 ml) and the other compartment contained ^{14}C -NANA (20 μl) in 0.5 ml PBS. The equilibrium dialysis cell contained 12 such pairs of compartments and contents of each pair at a time at various time intervals were removed, and their radioactivity content determined. PBS compartment (---●---), sample compartment (—○—).

To construct a binding curve (Figure B.7), the radioactivity content in the antiserum compartment was determined after 60 hours of equilibration.

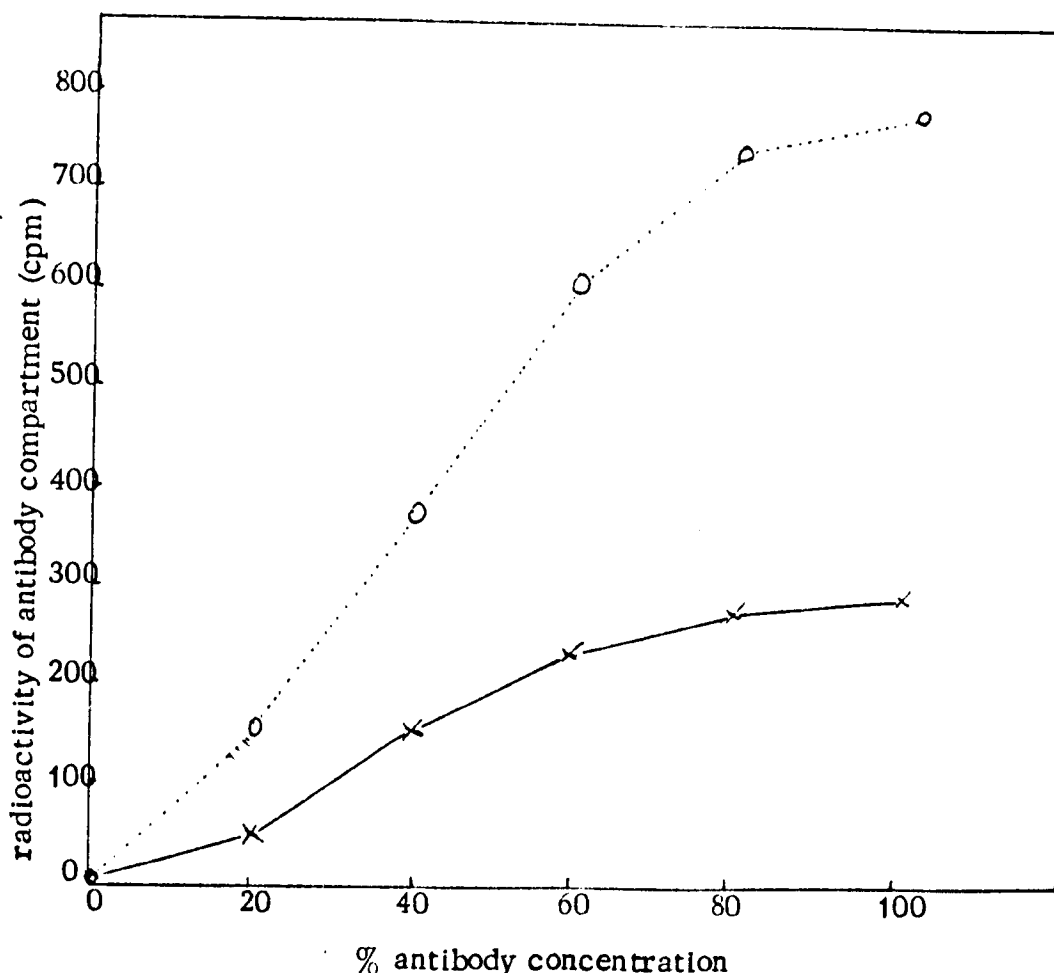


Figure B.7 ^{14}C -NANA binding by F_{Ab} (—x—x) and C_{Ab} (····o····) as determined by equilibrium dialysis. ^{14}C -NANA solution (20 μl) was added in each of the compartments containing PBS or various dilutions of F_{Ab} , C_{Ab} or control serum. The values plotted were corrected for non-specific binding of ^{14}C -NANA by subtracting the counts of the PBS-compartment and of the corresponding dilution of control serum from the counts in the F_{Ab} or C_{Ab} compartments. The dilutions were calculated, taking the 5mg/ml working solution of antibody solution as 100%.

(e) Competition of ^{14}C -NANA and unlabelled NANA for C_{Ab}

The antiserum to colominic acid showed a good dose-response curve in its binding of ^{14}C -NANA and appeared to bind more ^{14}C -NANA than did the antiserum to fetuin. The immunoglobulin fraction prepared from

colominic acid antiserum was used to assess the possibility of using it in a competition assay of sialic acid. Figure B.8 shows the competition of varying amounts of unlabelled NANA against a constant amount of ^{14}C -NANA for a constant amount of C_{Ab} .

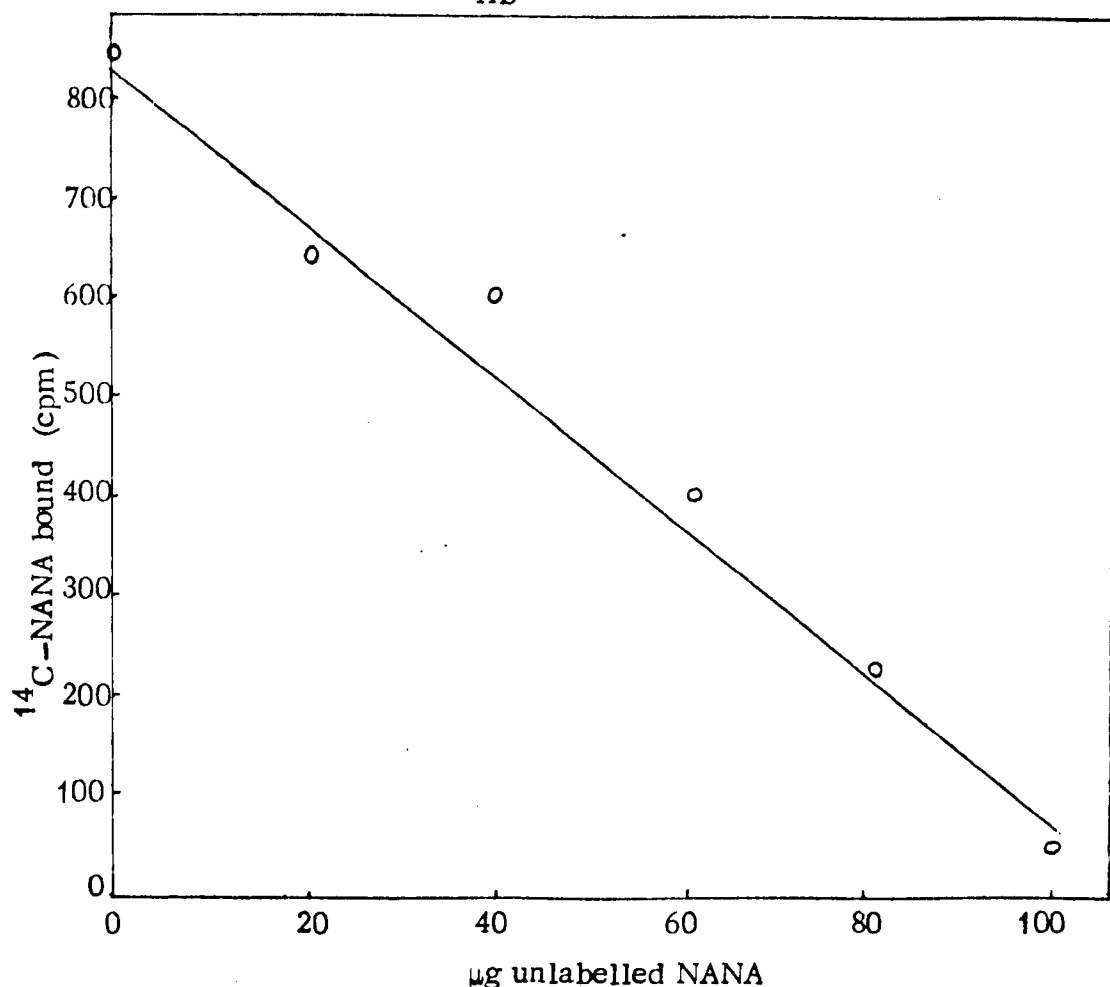


Figure B.8 Competition of ^{14}C -NANA and unlabelled NANA for C_{Ab} . The test compartments contained ^{14}C -NANA solution (20 μl), C_{Ab} (5 mg/ml in PBS, 0.5 ml) and varying amounts (0-100 μg) of unlabelled NANA in 100 μl PBS. The ^{14}C -NANA bound by C_{Ab} was determined as described earlier.

Similar experiments were repeated, using N-acetylgalactosamine, N-acetylglucosamine, colominic acid, D-mannose, D-glucose or D-galactose instead of unlabelled NANA (Figure B.9).

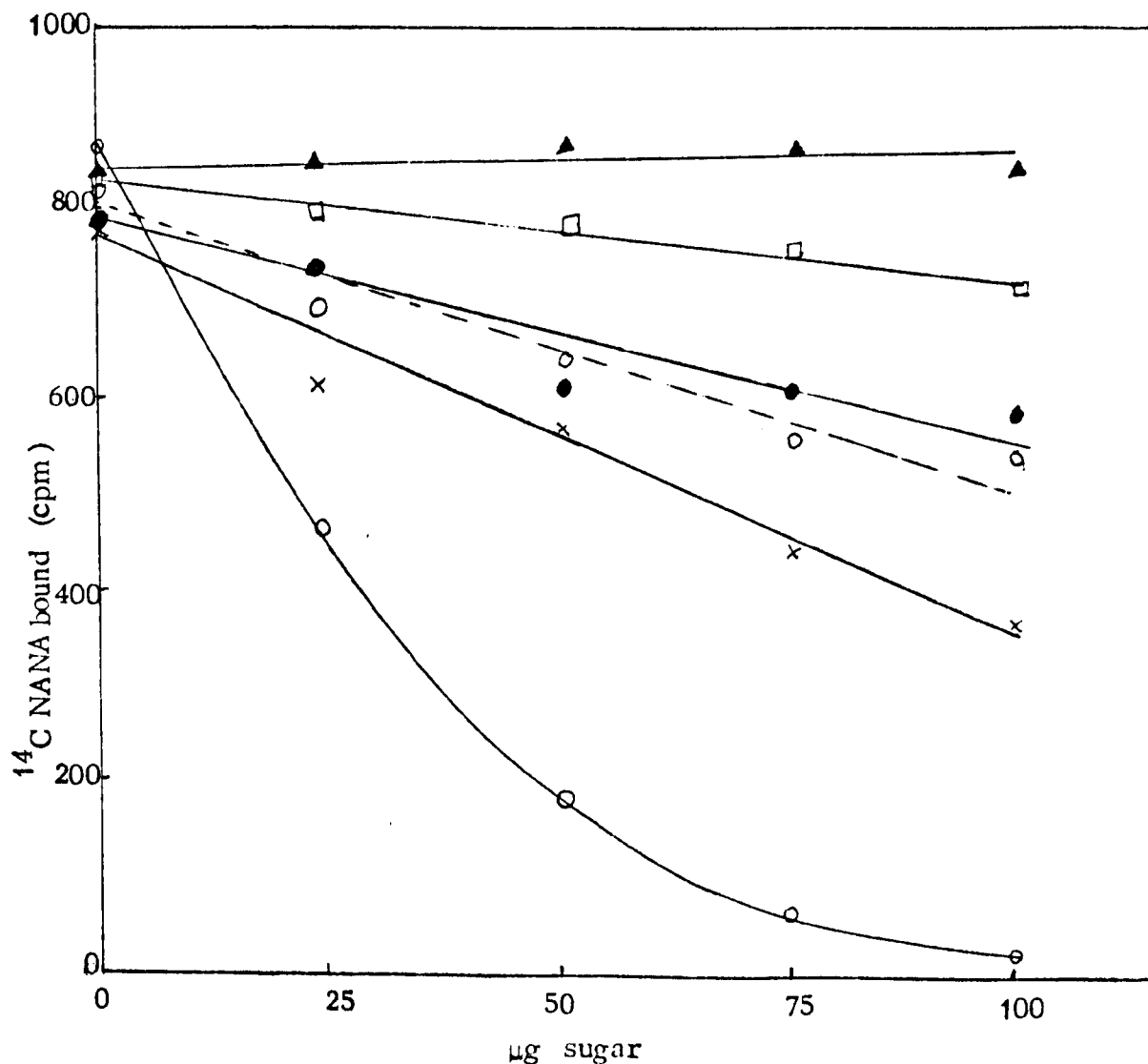


Figure B.9 Competition of ^{14}C -NANA and other sugars for C_{Ab} . The test compartment contained ^{14}C -NANA solutions ($20\ \mu\text{l}$), C_{Ab} ($5\ \text{mg/ml}$ in PBS, $0.5\ \text{ml}$) and the varying amounts of each of the sugars indicated: N-acetylgalactosamine ($\times\text{---}\times$), N-acetylglucosamine ($\bullet\text{---}\bullet$), colominic acid ($\circ\text{---}\circ$), D-mannose ($\circ\text{---}\text{---}\circ$), D-glucose ($\blacktriangle\text{---}\blacktriangle$) and D-galactose ($\square\text{---}\square$).

(f) Attempts to purify C_{Ab}

The immunoglobulin fraction, C_{Ab} , was shown to bind to a column of protein-A Sepharose previously equilibrated with PBS. C_{Ab} was eluted by $0.1\ \text{M}$ glycine-hydrochloric acid buffer, pH 2.8. The eluted protein fractions were pooled and concentrated by Ultrafiltration through a PM 10 membrane. The concentrated C_{Ab} fraction was then passed through a

column of starch-colominic acid, prepared as described by Uchida *et al.* [208] but equilibrated with PBS and eluted with 0.1 M glycine buffer, pH 2.8. The eluted C_{Ab} protein fraction had the same properties as the serum obtained from colominic-acid-injected rabbits using the immunological techniques described above.

DISCUSSION

Contrary to previous reports that colominic acid lacks serological properties, the results of the present study suggest that when colominic acid is injected in rabbits, a component is produced in low concentration in serum which tightly binds ^{14}C -NANA as attested by the Farr assay and analysis by equilibrium dialysis. However, other conventional techniques for antibody detection, *e.g.* immunodiffusion precipitation, failed to demonstrate such a component. Since the component binds to protein-A, it is an immunoglobulin and since no similar component is detected in control serum, one could conclude that colominic acid is immunogenic in rabbits. The fact that immunoprecipitation techniques fail to detect the antibodies produced may relate to the nature of the complexes formed between colominic acid and the antibody molecules. Since immunoglobulins contain sialic acid [303], it is possible that secondary interactions, normally responsible for stabilising antigen-antibody complexes, are prevented by repulsive interactions between the sialic acid moieties of the antibody molecules and those on colominic acid. The specificity of the C_{Ab} component is further demonstrated by its ability to bind on to a starch-colominic acid column, a property not shared by the control serum.

For the purposes of radioimmunoassay for sialic acid, the good dose-response obtained with C_{Ab} would indicate its potential use. However, the rather high competition afforded by sugars such as N-acetyl-D-glucosamine, D-mannose and D-galactose in inhibiting the binding of ^{14}C -NANA to C_{Ab} is a big limitation for the practical application of this technique to assay sialic acid in biological samples. Other applications of antibodies to sialic acid such as labelling of cell surfaces, etc. would interest many who are researching on the biological importance of this sugar.

APPENDIX III

Design of a longitudinal gel-slicer

Disc electrophoresis on cylindrical gels is attractive to many investigators because of its sharp, well-defined separation boundaries which can be achieved with only minute quantities of sample. After successful separation of the sample components, it is often desirable to compare the area of enzymic (or other biochemical) activity with the corresponding protein (or other component) fraction. An additional sample is usually run for that purpose. Alternatively, the same sample gel stained for enzymic activity can, after appropriate washing, be subsequently stained for protein and the resulting colour quality is assessed for the presence of both enzyme and protein, if they contrast with other protein bands.

However, it has often been necessary to obtain longitudinal sections of cylindrical gels after electrophoresis, which can then be treated differently and compared, because of several reasons, including: (a) attempt to avoid exposing the gel and sample components to the deleterious chemical reactions and other incompatible treatments associated with their localisation, (b) the need to correct for differences arising from dissimilarities in extents of gel-cross-linkage and actual gel dimensions, (c) sample and gel material economy, (d) correction for artefacts introduced by differences in sample loading and separation, etc. The methods employed in obtaining longitudinal gel-slices have so far included slicing the gel with razors, scalpel or thin wire guided by the hand, all procedures which involve considerable handling problems and gel distortion. Some of these slicing methods often require prior treatment, e.g. embedding or hardening of the gel. In this section, a simple device (Fig. C.1) inexpensively designed to obtain identical longitudinal sections of cylindrical gels is described and its advantages and applications are discussed.

The device was constructed out of perspex and consists of a cylindrical shaft, E, of appropriate diameter, into which the gel-containing tube, D, fitted tightly when it was inserted. Before the gel tube was inserted, the

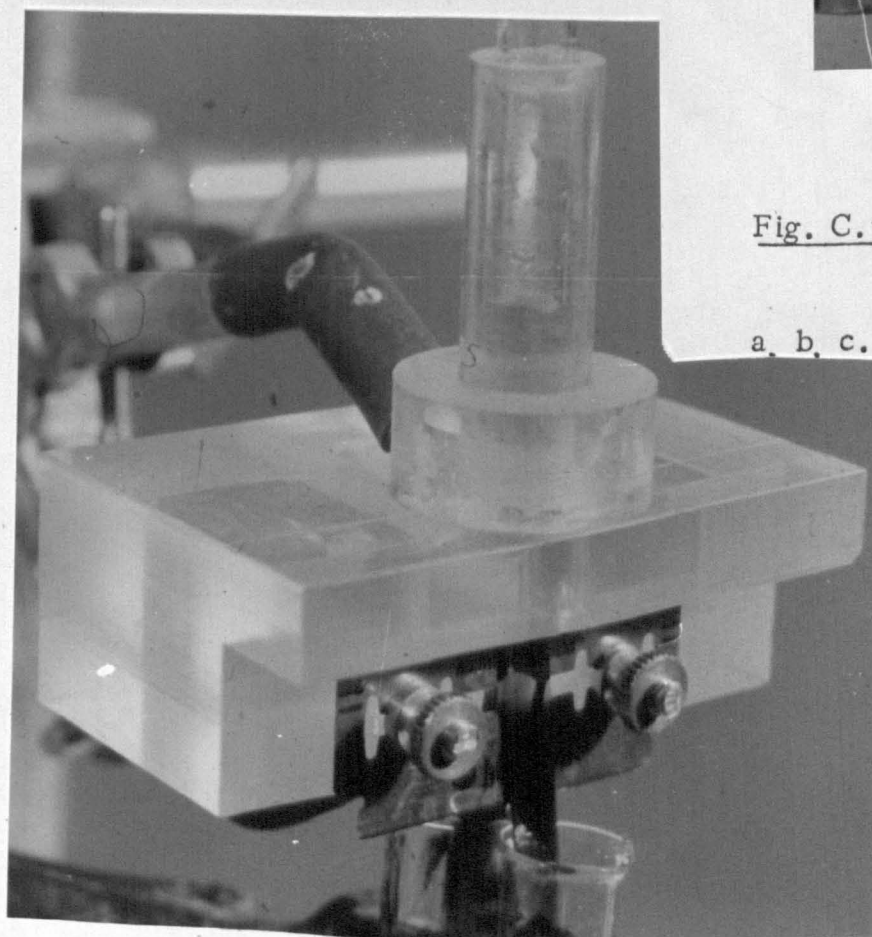
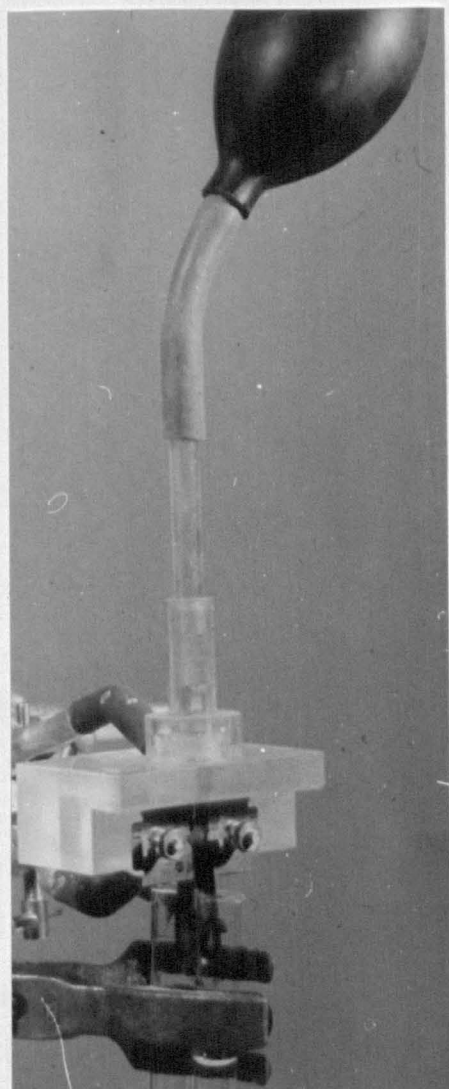
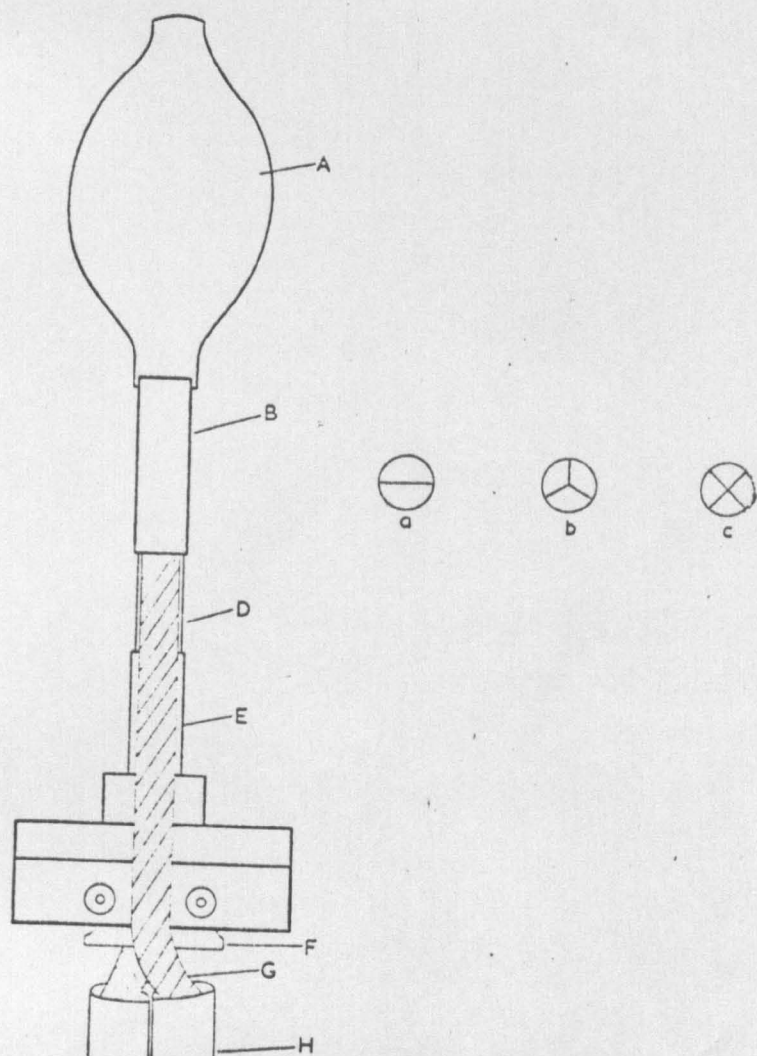


Fig. C.1 Longitudinal section of the slicer. a, b, c

a, b, c. Transverse section of cutting edges used to produce 2, 3 and 4 longitudinal sections respectively.

gel was loosened from the tube by carefully squirting water between the gel and the tube with a fine needle syringe [218] to ensure free movement of the gel which is essential for accurate sectioning. The gel tube was then inserted until its bottom rested firmly on a horizontal, sharp thin blade, F, lying across the diameter of the shaft and gel tube. A short section of rubber tubing, B, was attached at the free end of the gel tube, and filled with water; a bulb equipped with an air valve (or a syringe), A, was attached on to the tubing as shown in Fig. C.1.

The gel moved down the tube smoothly by hydraulic transport when the bulb or syringe was squeezed gently and its lower end was smoothly sliced as it got forced onto the razor. The resulting gel slices, G, were conveniently collected in receivers, H, placed underneath the cutting blade.

The precision, reproducibility and uniformity of slicing were checked by weighing and matching the slices from gels contained in ten tubes of approximately similar dimensions, using polyacrylamide total gel concentrations in the range 3.5% - 20.0% and gel tube diameters of 0.3, 0.4 and 0.5 cm.

DISCUSSION

The development and improvements of transverse gel-slicers are commonly reported [304], but the problems of obtaining uniform longitudinal gel-slices have often either been ignored or left to individual practical solution and originality. The longitudinal gel-slicer described is simply constructed and simplifies slicing operations, affording rapidity and uniformity over a wide range of acrylamide concentrations. Its advantages include sample economy, slicing directly from the electrophoresis tube without prior treatment of the gel, among others. The device can be adapted to slice gels from tubes of varying diameters and to produce more than two slices, as needed, by utilising the appropriate number of cutting edges (Fig. C.1, a, b, c).

Its applications include comparison of different functions of a component after electrophoresis to facilitate accurate identification of electrophoresis bands. This application was described in Chapter 2 when staining neuraminidase with protein or glycoprotein stains. The device is also useful for conveniently producing longitudinal slices that are required in various methods of detecting enzymes on gels after electrophoresis, e.g. the zymogram technique and other methods [305].

APPENDIX IV

Sources of materials

N-acetylgalactosamine	Sigma, London
N-acetylglucosamine	Sigma, London
N-acetylneuraminic acid	Sigma, London
N-acetyl[4, 5, 6, 7, 8, 9- ¹⁴ C]neuraminic acid	The Radiochemical Centre, Amersham
α-acid glycoprotein	Scottish National Blood Transfusion Association, Edinburgh
Black K salt	Koch Light Laboratories Buckinghamshire
N-bromosuccinimide	Pierce and Warriner (UK) Ltd., Cheshire
Carbonic anhydrase	Sigma, London
Carboxymethyl cellulose (MC)	Whatman Ltd., Maidstone, Kent
Colominic acid, sodium salt	Sigma, London
Cyanogen bromide	Eastman Kodak Co., Rochester, N.Y.
Dansyl amino acid standards	Sigma, London
Dansyl chloride	Sigma, London
Dansyl hydrazine	Sigma, London
DEAE-cellulose	Whatman Ltd., Maidstone Kent
Diethyl pyrocarbonate	Sigma, London
5, 5'-dithiobis(2-nitrobenzoic) acid (DTNB)	B.D.H. Chemicals Ltd., Poole, Dorset
1-ethyl-3-(3-dimethylaminopropyl)carbodiimide	Sigma, London

N-ethylmorpholine	B.D.H. Chemicals Ltd., Poole, Dorset
^3H -glycine	The Radiochemical Centre, Amersham
Glycine ethylester hydrochloride	Sigma, London
Hydroxylamine hydrochloride	B.D.H. Chemicals Ltd., Poole, Dorset
Hydroxylapatite	Bio-Rad Laboratories, Kent
2, (3-methoxyphenyl)- α_1 -N-acetyl neuraminic acid (MPN)	Boehringer, Mannheim
Molecular weight markers	B.D.H. Chemicals Ltd., Poole, Dorset
Mucin	Sigma, London
Neuraminidase from <u>Cl. perfringens</u>	Sigma, London
2, 4-pentanedione (acetyl acetone)	B.D.H. Chemicals Ltd., Poole, Dorset
Phenylglyoxal	Koch Light Laboratories Buckinghamshire
PM-10 membranes	Amicon Corporation, Lexington, Mass., U.S.A.
Protein-A Sepharose	Pharmacia Fine Chemicals, Sweden
Rose Bengal	B.D.H. Chemicals Ltd., Poole, Dorset
Sephadex G-25	Pharmacia Fine Chemicals, Sweden
Sephadex G-100	Pharmacia Fine Chemicals, Sweden
Sepharose 4B	Pharmacia Fine Chemicals, Sweden
Sialyllactose	Sigma, London
Sodium borohydride	Hopkins and Williams, Essex
Thiobarbituric acid	Sigma, London
2, 4, 6-trinitrobenzenesulphonic acid (TNBS)	Sigma, London

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APPENDIX V

Published papers

**APPENDIX
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